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(54) Title: METHODS AND COMPOSITION FOR THE MODULATION OF HOST IMMUNE RESPONSE TO ALLERGENS (57) Abstract <p>The present invention provides novel compositions and methods useful in the modulation or selective suppression of host immune responses to an immunogen of interest, particularly to immunogens which are exogenous antigens or allergens. Subject compositions include antibody, antibody derived, and antibody-like molecules of primary antigen reactivity with respect to the immunogen of interest. Antibodies or antibody-like or antibody-derived molecules include antibody fragments such as Fab, and complementarity determining region peptides (CDRs) which may be grafted into a framework region of any species, particularly human. They also include human antibodies, derived from sensitized human lymphocytes produced by cell fusion with heterohybridomas, or by DNA cloning and expression. Other compositions include T cell receptor (TCR) molecules, obtained either from T cell clones or hybridomas or as purified TCR preparations. Immunoreactive peptides corresponding to some or all of the complementarity determining regions or hypervariable regions of the TCR are also employed.</p>		

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METHODS AND COMPOSITION FOR THE MODULATION OF HOST
IMMUNE RESPONSE TO ALLERGENS

1. Field of the invention

5 The invention relates generally to the field of immunology, and more particularly to methods and compositions for the modulation of the host immune response to immunogens, especially exogenous antigens such as allergens. The invention employs antibodies and
10 antibody-derived molecules, including T cell receptors (TCRs), to down-regulate the immune response to these antigens.

2. Background of the invention

15 **2.1 Allergic diseases**

 The overall patterns of immune responses to allergens indicate that T lymphocytes are central in the generation of allergic diseases (Romagnani, 1992, Immunol. Today, 13:379-380; O'Hehir et al., 1991, Ann. Review Immunol., 9:67-95). Following allergen processing
20 and presentation by antigen presenting cells (APC) they function as helper T cells in cooperating with B cells to produce antibodies such as immunoglobulin IgE. Receptors on mast cells and basophils bind allergen specific IgE.
25 Subsequent exposure to allergen results in the release of inflammatory molecules which cause allergic symptoms.

 Allergen sensitized T lymphocytes also function directly in initiating allergic responses. This includes the release of cytokines such as interleukin 5, so
30 promoting eosinophil mediated allergic responses in asthma and related diseases. They also initiate local inflammatory responses such as those caused by exposure to contact sensitizing allergens.
 Environmental sources of contact sensitizing allergens
35 include naturally occurring compounds such as

alkylcatechols present in poison oak and ivy as well as pollutants and industrial chemicals.

2.1.1 IgE-mediated diseases: Asthma,
allergic rhinitis and atopic
dermatitis

5 Classic immediate type allergic reactions are mediated by the IgE subclass of antibodies, and are directed against a variety of environmental allergens. The most common of these world-wide are dust mite, pollen
10 from grasses, trees, broad-leaf weeds, mold spores, and animal dander. After initial sensitization, exposure results in an immediate reaction caused by the release of a range of inflammatory molecules including histamine and prostaglandins from mast cells. A second wave of
15 cellular activation occurs several hours later, manifest in the skin as vasculitis with leukocyte infiltration, and in the lungs by eosinophil and lymphocyte infiltration. The second phase reaction is thought to be responsible for promoting the chronic changes and
20 hypersensitivity which often accompanies the allergic diseases.

2.1.2 Dust mite allergens

Over the last several years, evidence of the strong
25 involvement of dust mite antigen (DMA) allergy in allergic rhinitis and especially asthma has strengthened. In a recent study (Gergen and Terkeltaub, 1992, J. All. Clin. Immunol., 90:579-588) it was shown that allergic rhinitis without asthma was associated with skin test
30 reactivity to only three allergens, dust mite (house dust), ragweed, and rye grass. Asthma alone was only associated with reactivity to house dust and alternaria. Additionally many reports have documented the close physical association between dust mites in the home and
35 asthma (Sporik et al., 1992, Clin. Exp. All., 22:897-906;

Platts-Mills, 1992, J. All. Clin. Immunol., 89:1046-1060).

There are two major species of dust mite, *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). A range of 30 proteins have been identified in allergenic preparations derived from each species; but only two, group I, Mw 25,000, and group II, Mw 14,000 are recognized as the major mite allergens. Within each group, proteins are highly cross reactive between the two species. These two proteins account for 60-70% of the reactivity of the IgE in allergic individuals.

2.1.3 Rye grass allergens

Grass and weed allergens are important causes of allergic rhinitis and asthma. Most of the grasses are cultivated agriculturally or ornamentally and are prevalent in residential areas. Only about a dozen of more than 5000 species of grasses are important allergens since many of the grasses do not produce abundant pollen. Important allergenic grasses include Rye, Timothy, Kentucky blue and June. Many of the allergens of different species cross react antigenically, and show amino acid sequence homology and may share T and B lymphocyte epitopes.

Rye grass (*Lolium perenne*) which is widely distributed in temperate zones induces allergic reactions in pollen sensitive individuals. Five groups of allergens (*Lol p I*, *II*, *III*, *IV*, and *V*), some in multiple forms, have been identified (Marsh et al., 1987; Thomas, 1991; Mathiesen et al., 1991). *Lol p I* is a major allergic species. Elevated levels of *Lol p I* specific IgE have been found in sera of up to 95% of grass pollen-allergic subjects (Freidhoff et al., 1986, J. All. Clin. Immunol., 78:1190-1201). *Lol p I* is an acidic glycoprotein of molecular weight 34 kDa, located in the

cytosol of the pollen. Certain IgE immunodominant epitopes have been identified. For example, a C-terminal peptide (aa 213-240) obtained by tryptic digestion of *Lol p I* has been found to contain an IgE binding site.

- 5 Synthetic peptides from the *Lol p I* C terminus (aa 216-240) also contain IgE and IgG binding sites (Van Ree et al., 1992).

2.1.4 TC mediated allergic skin disease: Poison oak and ivy dermatitis

- 10 Poison oak and ivy dermatitis is one example of a direct T cell mediated allergic reaction. This reaction occurs against the hapten urushiol when it contacts the skin, bronchial epithelium or the like. The hapten is a
15 mixture of 3-n-alkylcatechols found in the oil of the plant, which conjugates to body proteins. The conjugate stimulates T cells to directly attack the skin or other cells to which the hapten is bound.

- T cell etiology of the disease has been confirmed by
20 studies in animal models of poison oak dermatitis which include the guinea pig and mouse. Transfer studies in animals have demonstrated the allergic reaction can be transferred from sensitized to naive animals by T lymphocytes. Human and animal studies have identified
25 the specificity of the T cell reaction against the allergen. Both the specificity and antigenicity of the compounds reside primarily in the common catechol structure. The immunologic reactions seen with urushiol are typical of those caused by various identified contact
30 sensitizers, such as chlorinated hydrocarbons and epoxy resins.

- In humans, oral hyposensitization is quite effective although cumbersome since it requires 3-6 months of daily
35 administration of the oil. Although antibodies against urushiol have never been directly demonstrated, the antibody fraction of sera from desensitized patients will

transfer tolerance to mice, suggesting a role for regulatory antibodies.

2.2 IgG mediated response to protein antigens: RTA

5 There are other cases in which undesirable immune responses occur to protein antigens, such as ricin A chain (RTA) a protein which, when coupled to a targeting agent such as a lymphokine or monoclonal antibody such as mAb 791T/36 (immunotoxin), directed against colorectal
10 cancer, can kill the targeted cell. RTA is a very potent immunogen, and a single injection of immunotoxin in animals or humans produces a vigorous IgG immune response against all parts of the molecule.

15 2.3 Immunology of allergic diseases

 T lymphocytes, predominantly the CD4+ subset (Th2), play a central role in the initiation and maintenance of aero-allergen-mediated immune responses (Peltz, 1991; O'Hehir et al., 1991; Romagnani, 1992). Following
20 exposure of susceptible subjects, T lymphocytes collaborate with B lymphocytes in producing cytokines. These interact with T and B cells, as well as other cells of the immune network such as macrophages. The lymphokines stimulate the enhanced production of helper T
25 cells and B cells. Helper T cells further differentiate into two distinct subsets (Th1 and Th2) with characteristic lymphokine profiles. Th2 cells produce interleukin 4 (IL-4), which is one signal required for IgE antibody synthesis. A second signal for IgE
30 synthesis is produced following cognate T-B cell interactions involving recognition of T cell receptor/CD3 complex and major histocompatibility complex class II molecules (Romagnani, 1990; Geha, 1992).

 In addition to their role in IgE production, Th2
35 lymphocytes produce other lymphokines, particularly interleukin 5 (IL-5), which are required for eosinophil

recruitment and for initiating eosinophil mediated toxic responses (Clutterbuck et al., 1988, Blood, 73:1504; Lopez et al., 1988, J. Exp. Med., 167:219). IL-5 also-promotes most cell degranulation with release of

5 cytokines, basic granule proteins, and platelet - activating factor. The T lymphocyte is critical for both immediate and late phase reactions, but is more directly involved in the late phase. This is demonstrated by the fact that steroids are active in preventing the late

10 phase, but not the immediate phase reaction.

This overall pattern of immune responses to allergens implicates T cells as pivotal in the generation of immunoglobulin E and eosinophilia, two major components of an allergic response to aeroallergens

15 (O'Hehir et al., 1991, Ann. Rev. Immunol., 9:67-95). Control of T cell responses to allergens through intervention at any of several points in the immune pathway therefore constitutes a major approach to treating allergic diseases. This is illustrated by the results from treatment of severe chronic asthmatics with

20 cyclosporin A. Cyclosporin A is a potent immunosuppressive agent which acts principally on T lymphocytes. Patients treated with cyclosporin A showed a clinically significant improvement in their asthma

25 symptoms.

2.4 Immunotherapy with allergens

Immunotherapy of allergic diseases including asthma, allergic rhinitis, as well as urushiol dermatitis has

30 been undertaken for many years using hyposensitization with crude allergen extracts. This form of immunotherapy is effective in many patients and can provide lasting benefit even after immunotherapy has been discontinued (Busse, 1988, J. All. Clin. Immunol., 82:890-900).

35 Immunotherapy with grass extracts has proven useful in treating allergic rhinitis and asthma (Creticos and

Norman, 1987, JAMA, 258:2874-2880; Creticos, 1989, J. All. Clin. Immunol., 83:554-562; Reid et al., 1986, J. All. Clin. Immunol., 78:590-600). In like fashion, patients with severe asthma achieved significant improvement following treatment with DMA-antibody complex (Machiels et al., 1990, J. Clin. Invest., 85:1024-1035), also clinical improvement and decreased bronchial sensitivity on bronchial provocation in children immunized for three years with a house dust extract (Creticos and Norman, 1987, JAMA, 258:2874-2880, Creticos, 1990, J. All. Clin. Immunol., 83:554-562; Bousquet et al., 1985, J. All. Clin. Immunol., 76:734-744), and in patients with allergic rhinitis desensitized with either purified AgE from ragweed, or with the whole extract (Norman et al., 1968, J. All., 42:93-108). Similarly, with poison oak/ivy urushiol, oral hyposensitization is quite effective although cumbersome since it requires 3-6 months of daily administration of the oil. Indirect evidence for a regulatory role for anti-idiotypic antibodies and/or regulatory T cells is provided by several experimental and clinical studies. Subjects hyposensitized with urushiol generate immunoglobulins which can transfer tolerance to mice (Stampf et al., 1990, J. Invest. Derm.). However, there are numerous difficulties with this form of treatment. Allergen extracts are crude, so that treatment schedules are not able to be standardized. Also, prolonged courses of treatment result in low patient compliance. Since the precise immune mechanism activated by hyposensitization with allergic extracts is not known, the cause of therapeutic failures usually cannot be established.

Several mechanisms may be postulated whereby immunoregulation of T cell dependent allergic responses can be achieved. One mechanism is to interfere with antigen processing and presentation to the T cell. Another is to tolerize the T cell, either directly or by

production of T suppressor cells. This process will block either cognate T-B cell interactions or production of cytokines.

Indirect evidence for a regulatory role for anti-idiotypic antibodies and/or regulatory T cells is provided by several experimental and clinical studies. Subjects hyposensitized with urushiol generate immunoglobulins which can transfer tolerance to mice (Byers et al., 1990). In immediate type hypersensitivity, a general finding during hyposensitization is an initial increase in IgE antibodies, followed by a decrease. Concomitantly, there is an increase in the allergen specific IgG (Creticos, PS, JAMA, 268:2834-2839). When the specificity of the response is investigated carefully, it is found that auto-anti-idiotypic antibodies develop (Gurka et al., 1988, Ann. Allergy, 61:239-243). This has been shown to be the case in rye grass hyposensitization as well. Clinically, anti-idiotypic antibodies are elevated in human subjects following hyposensitization with rye grass extracts concomitant with reduction in allergic rhinitis (Hébert et al., 1990, Clin. Exp. Immunol., 80:413-419).

2.4.1 Immunotherapy with anti-idiotypic antibodies (Ab2)

While transfer of anti-idiotypic antibodies results in suppression of both T and B lymphocyte initiated response, immunotherapy with monoclonal or polyclonal anti-idiotypic antibodies has not been successfully developed for allergic diseases, since the responses in experimental systems have been contradictory. In one example, treatment of mice with polyclonal anti-idiotypic antibodies generated against a mouse monoclonal antibody reacting with a tobacco mosaic virus protein polypeptide suppressed the ability of mice to generate antibody responses to the decapeptide (Norton and Benjamini, 1987,

Cell. Immunol., 109:418-419). In another example, polyclonal anti-idiotypic antibodies produced by immunization of guinea pigs with polyclonal anti-benzylpenicilloyl antibody were shown to down regulate the anti-benzyl penicillin IgE antibody responses when given either pre or post sensitization (Wetterwald et al., 1986, Mol. Immunol., 23:347-356). Also, polyclonal anti-idiotypic antibodies to a monoclonal antibody which reacted with a decapeptide of tobacco mosaic virus suppressed the induction of antibodies (the class is unknown) to this decapeptide in mice, when administered prior to sensitization (Norton and Benjamini, 1987). Clinically, transfer of serum from bee keepers who had tolerated multiple bee stings into individuals who previously developed anaphylaxis to desensitization allowed the receipts to undergo successful desensitization (Bousquet, 1987, J. All. Clin. Immunol., 79:947-954), and it is shown that the transferred whole sera contained anti-idiotypic antibodies against bee venom (Boutin et al., 1993, J. All. Clin. Immunol. in press). This has been explained by transfer of Ab2s which down regulated the response. Recently it has been confirmed that in one patient successfully treated in this way, the transferred whole sera did contain anti-idiotypic antibodies against bee venom. A decrease in skin test reactivity also was observed after transfer (Hébert, 1991).

In general, however, treatment with monoclonal Ab2 antibodies has been undertaken to up-regulate responses to specific antigens. These include cancer and viral antigens (Fung et al., 1990, J. Immunol., 145:2199-2206; Raychaudhuri et al., 1990, J. Immunol., 145:760-767, reviewed in Kohler et al., 1989, Clin. Immunol. and Immunopath., 52:104-116). Several of these are in clinical trials, for example against cancer antigens (Robins et al., 1991, Can. Res., 51:5425-5429).

Consistent with these findings, vaccination with a monoclonal Ab2 directed against *Lol p I* allergen produced up-regulation of the IgE and IgG anti-*Lol p I* antibodies (Boutin et al., 1991, J. All. Clin. Immunol., 87
5 (abstract) page 197). In all cases the up-regulation has been explained by the induction of Ab3 antibodies.

2.4.2 Immunotherapy with Ab1 antibodies

Regulation of the immune response by Ab1s has been
10 investigated. Monoclonal antibodies against bovine serum albumin, given to virgin mice in the absence of adjuvant, could down-regulate the IgG response to later
immunization with that antigen (Eddy et al., 1987, J. Immunol., 138:1693-1698). Polyclonal antibodies against
15 sheep red cells administered presensitization could also nonspecifically down-regulate the IgG response to that antigen (Heyman and Wigzell, 1984, J. Immunol., 132:1136-1143). The authors suggested that this effect was due to nonspecific antigen blockade by the Fc portion of the
20 molecule. However, these studies did not demonstrate modulation of an established immune response.

Saint-Rémy et al. have shown in several publications (Machiels et al., 1990, J. Clin. Invest., 85:1024-1035; Saint-Rémy et al., 1988, Eur. J. Immunol., 18:1009-1014)
25 that autologous antigen-antibody complexes, made by adding antigen to sera from patients who were refractory to hyposensitization, could down-regulate the IgE response to a variety of allergic diseases including asthma and atopic dermatitis. After administration of
30 antigen-antibody complexes, the level of anti-idiotypic antibodies was increased; this was associated with clinical improvement.

The mechanism of action in these studies has not been well investigated. Multiple mechanisms are
35 probable, including nonspecific blockage of antigen processing via the Fc receptor, and antigen specific

blockage of antigen processing (Heyman et al., 1990, Immunol. Today, 11:310-313; Wiersma et al., 1989, Scan. J. Immunol., 29:439-448; Sinclair et al.; 1987, Immunol. Today, 8:7679). However it would appear that polyclonal
5 antisera can down-regulate the IgG response. The use of monoclonal antibodies to down-regulate the IgE response is considerably more complex, in part because of the difficulty in selecting the specificity of the monoclonal antibody.

10

3. Brief description of the figures

Figure 1 shows anti-RTA response in BALB/c mice treated with a single injection of 50 μ g of immunotoxin 791T/36-RTA, measured by ELISA.

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Figure 2 shows binding of three anti-RTA mAbs to either native RTA, heat treated RTA (56° C for 30 minutes) (H-RTA), recombinant RTA which is not glycosylated (rRA), or deglycosylated RTA produced by periodate treatment of the polypeptide to oxidize the
20 terminal mannose residues (dGA).

Figure 3 represents binding of two other anti-RTA mAbs to the same antigen preparations, with high reactivity to H-RTA and relatively poor reactivity to the other species.

25

Figure 4 shows the result on anti-RTA antibody titers of treating BALB/c mice with a single dose of 100 μ g delivered intravenously 24 hours after initial sensitization with 50 μ g of immunotoxin. Anti-RTA titers
30 were measured by an ELISA assay, and results are expressed as percent reduction compared to the controls, mice sensitized with immunotoxin and injected with comparable amounts of an irrelevant mAb, 365, which is directed against carcinoembryonic antigen.

35

Figure 5 shows the dose response effect of mAb 608/7, given at doses ranging from 25-200 μ g 24 hours after immunization with Immunotoxin.

Figure 6 shows the effect of injection of 100 μ g of mAb 608/7 given intravenously at times from 2 to 24 hours after immunization.

5 Figure 7 shows the effect of treatment with 100 μ g of mAb 608/7 upon the anti-RTA IgG response of BALB/c mice immunized with immunotoxin, and treated with either mAb 608/7 (solid bars) or an equivalent amount of irrelevant mAb 365 (anti-CEA).

10 Figure 8 shows the effect of pretreatment with mAb 608/7 (anti-RTA) or 365 (anti-CEA) given from day -7 to 0 prior to immunization with immunotoxin.

Figure 9 is a graph showing *in vitro* inhibition of binding of anti-Der p I monoclonal antibody 4C1 to Der p I by monoclonal antibody 2C7.

15 Figure 10 is a graph showing *in vitro* inhibition of binding of anti-Der p I monoclonal antibody 2C7 to Der p I by murine anti-2C7 polyclonal anti-idiotypic antiserum; polyclonal antisera to other anti-Der p I monoclonal antibodies (4C1, 5H6), or to anti-Der p II monoclonal antibody (6D6), or to anti-carcinoembryonic antigen
20 monoclonal antibody (337, included as a nonspecific control) were ineffective.

Figure 11 is a graph showing inhibition of binding of human serum IgE to Der p I by murine anti-Der p I
25 monoclonal antibodies 10B9, 2C7, 4C1, and 1B12; A355, A380, A368, A361, A362, and A385 represent different human IgE antisera from subjects with atopic dermatitis.

Figure 12 represents the suppression in ear thickness of mice immunized with Der p I, then treated
30 with three different doses of anti-Der p I mAb H11, at doses of 3 x 5 μ g, 3 x 10 μ g, or 3 x 20 μ g, in alum, at days 4, 7, and 14 after sensitization. Animals are then challenged by ear injection with 10 μ g DMA or with saline, on day 28. The bottom axis represents increase
35 in ear thickness.

Figure 13 represents an immunoblot profile of three monoclonal antibodies, all directed against rye Group I. The first, shown in immunoblot A, is mAb 290-A-167; the second in immunoblot B, is mAb 348-A-6; and the third, shown in immunoblot C, is mAb 539-A-6. Three antigen mixtures were used: rye group I from NIH (lane 1 in each blot), and crude rye grass extracts from Omega (lane 2) and Pharmacia (lane 3).

Figure 14 shows silver nitrate-stained SDS slab gel (7.5-15% run under reducing conditions) demonstrating the molecular weights of the rye Group I antigens. Lane 1 is molecular weight markers, and lanes 2 and 3 are rye Group I antigen purified by affinity chromatography with either 5 μ g/well (lane 2) or 10 μ g/well (3) respectively.

Figure 15 shows inhibition by unlabeled mAbs (■—■, 290A-167; □—□, 348A-6; and ▲—▲, 539A-6) of the binding of labeled 290A-167 (a), 348A-6 (b) and 539A-6 (c) mAbs to their homologous polystyrene-bound anti-ID Abs. 13A-12 (x—x), 3E12 (◆—◆), and 3E-3 (△—△) mAbs were used as negative controls. Each point represents the mean value of the three experimental determinations.

Figure 16 shows inhibition by anti-ID Abs (■—■, anti-ID of 290A-167; □—□, anti-ID of 348A-6; and ▲—▲, anti-ID of 539A-6 mAbs) of the binding of labeled 290A-167 (a), 348A-6 (b), and 539A-6 (c) mAbs to polystyrene-bound rye I. Normal rabbit IgG was used as negative control (x—x). Each point represents the mean value of three experimental determinations.

Figure 17 shows inhibition by anti-ID Abs (■—■, anti-ID of 290A-167; □—□, anti-ID of 348A-6, and ▲—▲, anti-ID of 539A-6) of the binding of labelled rye I to polystyrene-bound 290A-167 (a); 348A-6 (b), and 539A-6 (c) mAbs. Normal rabbit IgG was used as negative control

(x—x). Each point represents the mean value of three experimental determinations.

Figure 18 shows inhibition by anti-rye I mAbs [290A-167 (a), 348A-6 (b) and 539A-6 (c), solid bars] or their homologous anti-idiotypic Abs (hatched bars) of the binding of human IgE to polystyrene-bound rye I. The concentration of each mAb was 1 μ g/ml and for anti-idiotypic antibody, the concentration was 250 μ g/ml.

Figure 19 shows inhibition of binding of labeled anti-rye I mAbs (290A-167 \blacktriangle — \blacktriangle , 348A-6 \blacklozenge — \blacklozenge , 539A-6 \blacktriangle — \blacktriangle) to solid phase fixed rye I antigen by human auto anti-ID antibodies from three (A, B, C) sera depleted of anti-rye I antibodies. The inhibited bindings of mAbs were as follows: 290A-167 = 34,400 c.p.m., 348A-6 = 78,526 c.p.m. and 539A-6 = 67,006 c.p.m. [The binding of anti-Hbs mAb (3E3) as control to its antigen was 43,988 c.p.m. and was not inhibited by any auto-anti-ID antibodies.]

Figure 20 shows specific suppression of anti--allergen antibodies by administration of idiotypic antibody. Groups of mice were immunized by i.p. injection of various amounts (0.01-10 μ g) of 290A-167 (Id) or 10 μ g of unrelated mAb (5 mice/group). Blood samples were regularly drawn and tested for specific anti-*Lol p I* Abs in ELISA or PCA assays.

Figure 21 shows change in specific antibody levels over time. Animals were injected with mAb 290, or an anti-transferrin mAb of the same subclass as mAb 290, which does not cross react with that antigen; and *Lol p I* antigen using the scheme shown in Figure 11. Sera were drawn at 2 week intervals, and binding to mAb 290 was measured at a dilution of 1:100 by an ELISA assay as described.

Figure 22 shows anti-idiotypic antibody levels as a function of mAb 290 dose. Groups of five mice were injected with *Lol p I* antigen, and concentrations of mAb

290 or with 10 μ g of the anti-transferrin mAb, ranging from 0.01 to 10 μ g using the scheme pictured in Figure 20. The levels of anti-idiotypic antibodies reactive with mAb 290 were assayed at a dilution of 1:100 by an
5 ELISA assay as described in the text. The right axis represents mAb immunizing Dose (μ g).

Figure 23 and 24 depict specific inhibition of IgE and IgG antibodies by mAb 290. Groups of 5 mice were injected with 10 μ g of mAb 290 or unrelated mAb (anti-
10 transferrin) of the same subclass. After depletion of anti-idiotypic antibody activity, sera were either diluted 1/50 and assayed for anti *Lol p I* antibody of the IgE class, or diluted 1/100 and assayed for anti *Lol p I* antibody of the IgG₁ class. The mice had last been
15 immunized with *Lol p I* at week 9.

Figure 25 depicts the structure of urushiol, the series of alkylcatechols in poison oak/ivy, with a C15 or C17 side chain.

Figure 26 summarizes studies in which binding of radiolabelled mAb 991 to pentadecylcatechol (PDC) was measured in the presence of a variety of related
20 compounds. Maximal inhibition of binding was noted with PDC and the catechols; minimal with pentadecanoic acid, an analog of the side chain, or with resorcinol, and
25 analog of the catechol ring but with hydroxyl groups in different positions.

Figure 27 depicts results of experiments in which BALB/c mice were pre-treated with one or more doses of mAb 991, then sensitized to PDC, and challenged (by ear
30 painting) with the same compound.

Figure 28 depicts results of experiments in which mice are initially sensitized with PDC or urushiol, then injected with mAb 991, 21 days later, after which they are challenged on day 42 by ear painting.

35 Figure 29 depicts results of experiments in which sensitized BALB/c mice are injected with a variety of

different doses of mAb 991 in alum prior to challenge. In this case, the percent reduction in ear thickness over controls is plotted, with the background (nonspecific) swelling subtracted.

5

4. Summary of the invention

The present invention provides novel compositions and methods useful in the modulation or selective suppression of host immune responses to an immunogen of
10 interest, particularly to immunogens which are exogenous antigens or allergens. Subject compositions include antibody, antibody derived, and antibody-like molecules of primary antigen reactivity with respect to the immunogen of interest. Antibodies or antibody-like or
15 antibody-derived molecules include antibody fragments such as Fab, and complementarity determining region peptides (CDRs) which may be grafted into a framework region of any species, particularly human. They also include human antibodies, derived from sensitized human
20 lymphocytes produced by cell fusion with heterohybridomas, or by DNA cloning and expression. Other compositions include T cell receptor (TCR) molecules, obtained either from T cell clones or hybridomas or as purified TCR preparations.
25 Immunoreactive peptides corresponding to some or all of the complementarity determining regions or hypervariable regions of the TCR are also employed.

In preferred embodiments, antibody and antibody--derived molecules and TCRs are directed against dominant
30 epitopes of the allergen of interest. Allergens include haptens, such as urushiol and certain pharmaceuticals, as well as allergenic proteins such as those in dust mite allergens, mold spores, and pollen.

Allergens to be down regulated may be from
35 environmental sources, from food, from cosmetics, or from drugs and therapeutics. Immune responses to be down

regulated include, but are not limited to, T cell reactions such as those causing poison oak and ivy dermatitis, as well as those initiating IgE-mediated diseases such as asthma, allergic rhinitis and atopic dermatitis. An especially important class of allergens are those which are commonly airborne. Airborne allergens are a significant source of asthma and allergic rhinitis since subjects frequently are exposed to airborne allergens via inhalation into the lungs, bronchioles, and nasal passages, or by airborne contact with conjunctiva. IgE-mediated allergic responses such as asthma and allergic rhinitis and atopic dermatitis are regulated by the proposed methods, as well as allergic reactions mediated by IgG responses. IgG-mediated allergic reactions are especially important mediating in sensitivity to drugs and to injected allergens as exemplified by ricin A chain.

Compositions of the present invention are believed to suppress host immune response to the immunogen of interest, at least in part by stimulation of anti-idiotypic antibodies and by interactions with cells involved in antigen processing and stimulation of the immune network.

The present invention also provides methods for selective suppression of host immune response to an immunogen of interest by administering a compound of the invention. In a preferred embodiment, subject compositions may be administered to the host after the host has been sensitized to the immunogen, thereby down-regulating the host sensitivity. Composition also may be administered to the host prior to sensitization.

5. Detailed description of the invention

As used herein, an Abl or idiotypic molecule is an antibody, TCR, or antibody- or TCR-derived molecule which binds to or reacts with a primary immunogen or allergen.

An Ab2 or anti-idiotypic molecule is an antibody, TCR, or antibody- or TCR- derived molecule which binds to or reacts with an Ab1 or idiotypic molecule against the primary immunogen. Thus the Ab1 or idiotypic molecule
5 itself functions as a secondary antigen in that it can produce an anti-idiotypic response. Of particular relevance are Ab2 molecules which bind to the allergen binding site of the Ab1.

As used herein, the term "antibody-derived" molecule
10 refers to an antibody, an antibody fragment, a hybridized antibody or antibody fragment, or a chimeric molecule comprising an antibody binding domain and a heterologous polypeptide. Antibody-derived molecules of the invention contain at least a binding domain of an Ab1 against the
15 allergen of interest. The binding domain includes the complementarity-determining regions (CDRS) of the Ab1, joined to a framework region (FR). The FR may be a sequence from an animal or a human; replacement of animal FRs with human FRs produces a humanized binding domain.

20 The constant domains of an antibody from an animal may be replaced by corresponding domains from a human to produce humanized antibodies. Antibody-derived molecules of the invention comprise both humanized binding domains and humanized antibodies.

25 A TCR may be employed analogously to an antibody-- derived Ab1. A TCR which contains a binding site for an epitope of an allergen of interest is capable of functioning in a manner similar to an antibody-derived Ab1. A "TCR-derived" molecule refers to a TCR, a TCR
30 fragment, a hybridized TCR or TCR fragment or a chimeric molecule. Particular embodiments of TCR derived molecules incorporate the binding domain of a TCR, but may have other domains of the TCR deleted, or may be formed into a chimeric molecule with soluble domains from
35 other proteins, such as serum albumin. For convenience, references herein to "Ab1s" includes TCR-derived

molecules which bind to an epitope of the allergen of interest, unless the context precludes this meaning.

A preferred embodiment of the invention comprises antibody-or TCR-derived molecules which have been
5 isolated and purified. As used herein, the term "isolated" means a composition which is free of substantial or immunologically active amounts of serum components other than antibody - or TCR-derived molecules. In particular, isolated antibody-derived
10 molecules are substantially depleted in major serum proteins and cytokines or lymphokines. "Purified" antibody or TCR-derived molecules are depleted of molecules which do not bind to the allergen of interest; in particular, purified Ab1s are depleted of Ab2s and of
15 Ab1s directed to other antigens.

However, compositions of the present invention may comprise Ab1s to which non-immunologically active molecules have been added, e.g., serum albumin from the species to which the composition is to be administered.

20 A particular preferred embodiment comprises Ab1 antibody - or TCR-derived molecules which are substantially free of the allergen to which the Ab1 molecule binds. "Substantially free" means that the ratio of the number of antibody binding epitopes is at
25 least 10:1, preferably 100:1, more preferably 1000:1.

One category of exogenous antigens of interest is referred to as environmental allergens. As used herein, the term "environmental allergen" refers to bin allergen to which an animal, including a human, is exposed by
30 external contact, and includes dermal or conjunctival contact and inhalation of airborne allergens. Environmental allergens include both immunogenic macromolecules and haptens.

Particular environmental allergens of interest
35 include pollens. Pollen is wind-dispersed by many types of higher plants, including gymnosperms (e.g., pines and

cypresses), dicotyledonous angiosperms (including broadleaf trees such as elms and many common broadleaf weeds such as ragweed and plantain) and monocotyledonous angiosperms (e.g., the grasses). The methods presented
5 here are applicable to any pollen allergen. Other allergens include dust mite antigens consisting of proteins from the body and feces of the dust mite, found in house dust, mattresses and carpet, but capable of becoming airborne. They also include molds such as
10 *alternaria*. The methods presented here are applicable to any of these allergens.

A second category of exogenous allergens of interest is those derived from foods and food products. Numerous food components, particularly proteins, are known to
15 cause food allergies. Examples are proteins of wheat and related cereal grains, and of legumes such as peanuts. The allergenic proteins of wheat and peanuts have been isolated. Abs against allergenic wheat and peanut proteins are particular embodiments of one aspect of the
20 invention, e.g., compositions comprising Abs against food allergens. Administration of compounds comprising Ab1-derived molecules against food allergens are useful for suppression of food allergies.

A notable example is provided by wheat gluten and
25 related proteins. These are potent allergens in some individuals, and provoke IgG mediated reactions to wheat products in the diet. Administration of compounds comprising Ab1-derived molecules against food allergens are useful for suppression of food allergies. Compounds
30 and methods of the present invention provide for down-regulation of IgG reactions, as seen with the ricin A chain allergen which can be useful as a component of cytotoxic drugs. Another example of an immunogenic protein drug is provided by insulin. Diabetics receiving
35 either animal or recombinant human insulin often develop anti-insulin antibodies.

Other food allergens for which particular proteins have been implicated are summarized in Sampson et al., JAMA, 1992, 268:2840-2844.

5 A third category of exogenous allergens of interest is drug and therapeutic allergens. Prominent examples of drug allergens are the various penicillins and related β -lactam antibiotics. Allergic reaction to penicillins can be life-threatening. Administration of a composition comprising an Abl-derived molecule against a β -lactam
10 antibiotic is useful for suppression of antibiotic allergy.

A fourth category of interest are those haptens and low molecular weight chemicals such as urushiol (poison ivy/oak) or industrial and environmental chemicals
15 causing either contact dermatitis or pneumonitis.

The responses to be down-regulated can be those mediated by T cells, as exemplified by poison oak dermatitis, or IgE, exemplified by Dust Mite Antigen, or Rye Grass, or by IgG, exemplified by ricin A chain or Rye
20 Grass.

Ab1s useful in down-regulation of an allergic response may be of any antibody class and subclass, e.g., IgM, IgE, IgG₁, IgG₂, IgG₄, etc., and may include TCRs. Class identity is of secondary importance compared to the
25 characteristics of the binding domain. Ab1s are believed to act in part by stimulating the formation of Ab2s directed against the binding domain of the Ab1. Although the specific binding domain of the Ab1 therefore is critical, other portions of the Ab1 molecule may be
30 modified appreciably, including those portions of the antibody which determine class.

For similar reasons, fragments of the Ab1 are active, provided that the desired allergen epitope binding domain is present. Any of the common antibody
35 fragments which retain binding specificity may be used. Also, cloned "fragments," or partial antibody sequences

obtained by expression of abbreviated genes encoding the binding domain, are within the scope of the invention.

Furthermore, chimeric molecules in which the Ab1 binding domain is joined to other proteins or protein domains also are active immunoregulators. The chimeric protein "partner" to which the allergen binding domain is joined may be selected from a great variety of sequences. In some cases the binding domain from an antibody of one class may be joined to the constant regions of another class to produce a class-switched antibody. When the constant region is from a different species from that of the binding domain, the resulting chimeric antibody is partially species switched. This procedure may be used to create partially humanized antibodies which have lessened antigenicity when injected into humans.

In particular instances, a chimeric partner may be chosen because it imparts desirable solubility or localization characteristics. For example, an allergen binding domain may be joined to a collagen domain of the target species. The resulting chimera remains localized at the injection site due to binding of the collagen domain to extracellular matrix. In other instances a chimeric partner is chosen because it is itself immunologically active and acts as an adjuvant. Examples include antigenic domains from proteins of common bacterial pathogens.

Since immunoregulatory molecules of the invention do not require constant domains for activity, the Fc portion of the IgG constant domain is not necessary for immunosuppression. This result is counter to the suggestion in the work by Heyman et al., that the Fc domain is critical for immunosuppression.

5.1 Production of monoclonal antibodies

Murine monoclonal antibodies to the antigen of interest are prepared by methods well known to those

skilled in the art (Goding, *supra*). BALB/c mice are immunized with specific antigen preparations using a variety of protocols including use of immunological adjuvants such as complete and incomplete adjuvant.

5 Spleen cells are prepared from mice shown to be producing antibodies of the proper specificity, and fused with cells from murine myeloma cell lines such as Ps-NS1, Sp 2/0, X63-Ag8.654, NSO/1 or the like by established procedures (Kohler and Milstein, 1975, Nature, 256:495-
10 497; US Patent No. 4,376,110). Clones are selected by two rounds of limiting dilution. Hybridoma cells are then either maintained in culture or propagated in the peritoneal cavity of BALB/c female mice after prior injection of pristane (0.5 ml). Ascitic fluid is
15 harvested from mice 10 to 15 days after hybridoma cell injection and antibodies purified by passing the fluid through a protein A-Sepharose 4B column.

5.2 Production of humanized monoclonal antibodies

20 The most advanced technology for humanizing monoclonal antibodies has been termed CDR-grafting or reshaping (Riechmann et al., 1988, Nature, 332:323-327). In this scheme, the complementarity determining regions (CDRs) of a murine antibody are transplanted into the
25 corresponding regions in a human antibody. There are three CDRs in the heavy chains and three in the light chains, and they are the regions of the mouse antibody which binds to a specific antigen. The CDRs are identified by ascertaining the amino acid sequence of the
30 variable regions of the mouse monoclonal antibody to the sequences of all known mouse variable regions, and the sequences of all known human variable regions. These are obtained by reference to Kabat et al. (Sequences of
35 Proteins of Immunological Interest, 4th ed. US Department of Health and Human Services, US Government Printing Office, Washington, DC, 1987). This allows the division

of the variable regions of the murine monoclonal antibody into the framework regions (FRs) and the CDRs. Since the CDRs are unique for each antibody, and the framework is relatively homologous for all antibodies of a given

5 isotype, this comparison reveals the numbering of the amino acids comprising the CDR. In order to graft these CDRs onto a human framework, two basic methods can be used. The chimeric light and heavy chains can be constructed from the genomic DNA clones of the mouse

10 hybridoma, and then these variable regions can be linked to the human constant regions in expression vectors which contain the designated human constant regions. Often the 5' and 3' ends of the cloned cDNAs are modified by using the polymerase chain reaction (PCR). (Erlich et al.,

15 1988, Nature, 331:461-462). These vectors are then introduced into mammalian cells such as the cos cell line, and the supernatant fluids are analyzed to confirm retention of antibody binding. After this, PCR technology is used to insert the mouse CDRs into

20 available human framework regions, or the entire variable region may be sequenced based on the sequence of the mouse CDRs and known human framework regions. A range of human framework regions exist, and have been inserted into expression vectors. The available human framework

25 region which most closely mimics the amino acid sequence of the murine antibody is selected, since it would be predicted that the CDRs would find a better "fit" into such frameworks. These reshaped human variable regions are cloned into the expression vectors containing the

30 designated human constant regions, and the vectors are introduced into cos cells, either by electroporation in which DNA is added to cells such as cos cells, and the mixture pulsed with voltage (such as 1,900 volts, 10 minutes), incubated and washed. The supernatant is again

35 tested for presence of antibody which retains the binding of the initial antibodies. Often, during the design

process a few positions in the human framework regions will have been highlighted as critical positions. It may not always be clear whether it is best to retain the amino acids found at those positions, or whether they should be changed to those which were present in the initial murine antibody. Therefore, if binding is suboptimal, these changes can be made at a later date.

For optimal expression, (since cos cells, while easy to work with, are often not high antibody producers) the vectors are introduced into a different mammalian cell line such as CHO cells.

In other variations of this technique, the CDRs may be cloned directly, and inserted into vectors containing both the variable region framework, and the constant region framework region of the desired human antibody. These techniques, have several variables, among them the primers and vectors used, the cells used as expression systems, the human frameworks which most closely mimic the murine antibody framework, the ways by which this latter determination is made (either by simple comparison of the amino acid sequence, or by computer modeling of the predicted three dimensional structure of the murine antibody and matching it to the selected human framework which may in turn have amino acid changes) and the selection of the optimal final cell line to carry the DNA for the humanized antibodies. These techniques have been detailed extensively in many publications, among them: (Winters and Milstein, 1991, Nature, 349:293-299; Riechmann et al., 1988, Nature, 332:323-327; Hird et al., 1991, Br. J. Cancer, 64:911-914; Gassow and Seemann, 1991, Methods in Enzymology, pp. 99-121; Jones et al., 1986. Nature, 321:522-525; Verhoeyen et al., 1988, Science, 239:1534-1536; Riechmann et al., 1988, Nature, 332:323-327; Kamman et al., 1989, Nucl. Acids Res., 17:5404; Maeda et al., 1991, Hum. Antibod. Hybridomas, 2:124-133.

5.3 Chimerization-humanization of murine monoclonal antibodies

Monoclonal antibodies of murine origin are humanized by various methods so as to reduce the immunogenicity of the proteins with respect to the murine immunoglobulin component. One way involves production of chimeric antibody by linking cDNA molecule encoding the V region of the murine mAb to DNA encoding the human constant region. This can be effected using several approaches described in issued patents. The chimerization procedure involves:

- (1) Isolation of messenger RNA (mRNA) from mouse hybridoma cells, then cloning and cDNA production.
- (2) Preparation of a full length cDNA library from purified mRNA from which appropriate variable (V) region gene fragments, of the light (L) and heavy (H) chain genes can be identified, sequenced and made compatible with a constant (C) region gene segment.
- (3) Preparation of C region gene segment modules by cDNA preparation and cloning.
- (4) Construction of complete H or L chain coding sequences by linkage of the cloned specific immunoglobulin V region gene segments to cloned human C region segment modules.
- (5) Expression and production of chimeric L and H chains in prokaryotic and eukaryotic cells.

Detailed procedures are provided in Cabilly et al., US Patent 4,816,567 (28 March 1989); Taniguchi et al., Eur. Patent EP 171496 (19 February 1986); and Kudo et al., EP 184187 (11 June 1986); and publications including Cabilly et al., 1984, Proc. Natl. Acad. Sci., USA, 81:3273-3277; Morrison et al., 1984, Proc. Natl. Acad. Sci., USA, 81:6851-6855; Neuberger et al., 1985, Nature, 314:268-270; Tan et al., 1985, J. Immunol., 135:3564-3567; Sun et al., 1987, Proc. Natl. Acad. Sci., USA, 84:214-218; Lui et al., 1987, J. Immunol., 139:3521-3526;

and Horowitz, 1988, Proc. Natl. Acad. Sci. USA, 95:8676-8682).

5.4 Selection of Ab1s

5 Several criteria may be used to select a preferred monoclonal antibody against an allergen to be down-regulated. These include:

10 5.4.1 Demonstration that the selected monoclonal antibody recognizes a human IgE immunodominant protein in the allergen mixture in those cases in which the allergen is a mixture rather than a single component

Many native allergens are composed of mixtures of proteins, which may number more than 20.

15 Functionally, the majority of the human immune response which is clinically significant (IgE in the case of most aeroallergens) is directed against 1-4 of these proteins. An example is short ragweed, in which over 50% of the IgE response is directed against *Amb v I* (known in the
20 earlier literature as *AgE*). Another example is the *Lol p I* protein in Rye grass, in which up to 80% of the IgE reacts against this protein. A third is *Der p I* protein and *Der p II* protein in dust mite allergens, in which
25 these two proteins account for well over 50% of the IgE response.

To identify such proteins, immunoblotting may be used. In this procedure the crude antigen extract is subjected to SDS-PAGE to separate the proteins by
30 molecular weight. Then, sera from patients clinically reactive to the given allergen mixture are overlaid onto the separated proteins, which have been
electrotransferred onto nitrocellulose paper. After incubation, the strips are developed by addition of reagents such as peroxidase-labeled anti-human IgE sera.
35 This identifies the protein(s), and their relative contribution can be demonstrated by isolation of the

proteins, and absorption of the IgE from patient's sera. Identification of an IgG immunodominant epitope may be performed using antihuman-IgG serum, see Section 6.5.3.

5 5.4.2 **Demonstration that the selected
monoclonal antibody recognizes a
human IgE immunodominant epitope on
the protein**

 A range of murine or human monoclonal IgG or IgM
antibodies are made by standard methods defined elsewhere
10 in this document and well known to those skilled in the
art. This panel of antibodies is used to identify the
immunodominant epitope on the molecule by checking the
ability of each antibody to substantially inhibit the
binding of the human IgE or IgG containing sera to the
15 protein. Substantial inhibition is at least 20%,
preferably by 40%, more preferably by 50%, 60%, 75%, or
even 90%. Alternatively, the ability of the human IgE or
IgG containing sera to inhibit the binding of each
monoclonal may be tested. In the latter case, the
20 monoclonal antibody may be radiolabelled with ¹²⁵I, mixed
with the human serum, and added to polystyrene removable
strips coated with the protein. Determination of
radioactivity remaining after incubation and washing of
the wells will quantitate the amount of blocking, and
25 identify immunodominant epitopes. Usually such a protein
contains 2-6 immunodominant epitopes, and the monoclonal
antibodies reacting with these epitopes are selected for
further study.

30 5.4.3 **Demonstration that the selected
monoclonal antibody stimulates an
anti-idiotypic specificity similar to
that induced in humans, including
those receiving allergen specific
immunotherapy**

 In the case of allergens, humans subjected to
35 hyposensitization and enjoying clinical improvement of
their symptoms are available. The identification of the

specificity of the auto-anti-idiotypic antibodies in the sera of these patients also aids in identification of Abs likely to be effective in down-regulation of the IgE response, since anticipated they will be directed against the naturally occurring Ab1 which in turn identifies the appropriate epitope for down regulation. Therefore, one approach to identify the specificity of the candidate monoclonal Ab1s is to measure their ability to inhibit binding of ¹²⁵I labeled "candidate" monoclonal antibody to the protein. In one such procedure naturally occurring polyclonal human Ab1s are removed from the sera from hyposensitized humans by absorption with the antigen, so they will not interfere with the assay. They are also absorbed with normal murine IgG coupled to Sepharose CL4B, if a murine monoclonal antibody is to be tested. The sera, now containing only Ab2 antibodies against the protein in question, are then tested for the inhibition of binding of murine monoclonal anti-rye *Lol p I* monoclonal antibody to the antigen. The antigen is coated onto polystyrene removable strips, and the candidate monoclonal antibody, now radiolabeled, is mixed with the absorbed human sera, or incubated alone (as the positive control). Wells are washed and measured. Reduction of radioactivity confirms the percent of inhibition, and identifies the monoclonal antibody which recognizes the specificity of the auto-anti-idiotypic antibody present in highest amount.

In general, an antibody binds at least 20%, more preferably 40%, 50%, 60%, 75% or greater of the auto anti-idiotypic antibody induced in a hyposensitization subject.

35

5.4.4 Demonstration that treatment of animals with the mAb significantly down-regulates the immune response against the allergen

If an animal model exists, the ability of the selected monoclonal antibody(s) which may be used as a cocktail, to down-regulate the immune response may be tested. In this case, the animal may be sensitized either by injection of the allergen in adjuvant, without adjuvant, or in the aerosolized form. The candidate monoclonal antibody(s) are then injected before or at various periods after sensitization, and their ability to down-regulate either the primary response or the response to antigen challenge is measured. In most cases tested, the response is down-regulated by at least 50%.

These criteria are used to identify the optimal antibody for down-regulation of the IgE response.

The immune response to a wide variety of antigens or antigen mixtures can be down-regulated by the appropriate Ab1. In the most complex case, native allergens which are composed of a variety of proteins, the following line of investigation will aid in selecting the appropriate Ab1:

1. Identification of the immunodominant protein(s) in the mixture.

Although many native allergens are composed of mixtures of proteins which may be greater than 20; functionally the majority of the human immune response which is clinically significant (IgE in the case of most aeroallergens) is directed against 1-4 of these proteins. An example is short ragweed, in which over 50% of the IgE response is directed against *Amb v 1* (known in the earlier literature as AgE). Another example is the *Lol p I* protein in Rye grass, in which up to 80% of the IgE reacts against this protein. A third is *Der p I* protein and *Der p II* protein in dust mite allergens, in which

these two proteins account for well over 50% of the IgE response.

To identify such proteins, immunoblotting may be used. In this procedure the crude antigen extract is subjected to SDS-PAGE to separate the proteins by molecular weight. Then, sera from patients clinically reactive to the given allergen mixture are overlaid onto the separated proteins, which have been electrotransferred onto nitrocellulose paper. After incubation, the strips are developed by addition of reagents such as peroxidase-labelled human anti-IgE sera. This identifies the protein(s), and their relative contribution can be demonstrated by isolation of the proteins, and absorption of the IgE from patient's sera.

2. The immunodominant epitope(s) are then identified on the relevant protein:

A range of murine or human monoclonal IgG or IgM antibodies are made by standard methods defined elsewhere in this document and well known to those skilled in the art. This panel of antibodies is used to identify the immunodominant epitope on the molecule by checking the ability of each antibody to substantially inhibit the binding of the human IgE containing sera to the protein. Alternatively, the ability of the human IgE containing sera to inhibit the binding of each monoclonal may be tested. In the latter case, the monoclonal antibody may be radiolabeled with ^{125}I , mixed with the human serum, and added to polystyrene removable strips coated with the protein. Determination of radioactivity remaining after incubation and washing of the wells will quantitate the amount of blocking, and identify immunodominant epitopes. Usually such a protein contains 2-6 immunodominant epitopes, and the monoclonal antibodies reacting with these epitopes are selected for further study.

3. In the case of allergens, humans subjected to hyposensitization and enjoying clinical improvement of

their symptoms are available. The identification of the specificity of the auto-anti-idiotypic antibodies in the sera of these patients also aids in identification of Abs likely to be effective in down-regulation of the IgE response, since it is anticipated they will be directed against the naturally occurring Ab1 which in turn identifies the appropriate epitope for down regulation. Therefore, one approach to identify the specificity of the candidate monoclonal Ab1s is to measure their ability to inhibit binding of ¹²⁵I labeled "candidate" monoclonal antibody to the protein. In one such procedure naturally occurring polyclonal human Ab1s are removed from the sera from hyposensitized humans by absorption with the antigen, so they will not interfere with the assay. They are also absorbed with normal murine IgG coupled to Sepharose CL4b, if a murine monoclonal antibody is to be tested. The sera, now containing only Ab2 antibodies against the protein in question are now tested for the inhibition of binding of murine monoclonal anti-rye *Lol p I* monoclonal antibody to the antigen. The antigen is coated onto polystyrene removable strips, and the candidate monoclonal antibody, now radiolabeled, is mixed with the absorbed human sera, or incubated alone (as the positive control), and wells are washed and the reduction of radioactivity confirms the percent of inhibition, and identified the monoclonal antibody which recognizes the specificity of the auto-anti-idiotypic antibody present in highest amount.

4. If an animal model exists, the ability of the selected monoclonal antibody(s) which may be used as a cocktail to down-regulate the immune response may be tested. In this case, the animal may be sensitized either by injection of the allergen in adjuvant, without adjuvant, or in the aerosolized form. The candidate monoclonal antibody(s) is then injected before or at various periods after sensitization, and its ability to

down-regulate either the primary response or the response to antigen challenge is measured. In most cases tested, the response is down-regulated by at least 50%.

Obviously, if the clinically important allergen has only one protein, such as ovalbumin in chicken egg white or parvalbumin in cod fish, both of which induce an IgE reaction, or casein in cow's milk which induces an IgG reaction, or if the clinically significant allergen has only one epitope such as urushiol which induces a T cell response, this selection procedure may be shortened.

6. Example: AbIs to dust site allergens

6.1 Dust mite allergens

Dust mite allergy is responsible for a range of allergic diseases, principally asthma, allergic rhinitis and probably atopic dermatitis. Although these diseases can also be produced by a variety of other allergens, such as pollens, dust mite ranks as one of the top three offenders. The allergy is caused by exposure to mites of the genus *Dermatophagoides*, particularly *Dermatophagoides pteronyssinus* (*Der p*) and *Dermatophagoides farinae* (*Der f*). These mites are found worldwide, in house dust, mattresses, rugs, etc. so they produce symptoms year round. A range of at least 24 different proteins has been identified although *Der I* (molecular weight 24,000) and *Der II* (molecular weight 15,000) are generally recognized as the major mite allergens. These proteins have been sequenced and cloned, and it is now possible to determine which epitopes are responsible for reaction with T cells and IgE antibodies. There is considerable homology between the *Der I* antigens, and the *Der II* antigens, so that both species of dust mite produce similar symptoms, even though geographically one may dominate over the other. Individuals sensitized to dust mite allergens such as *D. pteronyssinus* produce IgG, IgA, and IgE antibodies. These persist in atopic individuals

and correlate with allergic symptoms. Although multiple subclasses of antibody may be produced, it is the IgE levels that are associated with allergic diseases.

5 Hyposensitization, one of the main methods of therapy against these diseases, results in an increase in IgG levels and a decrease in IgE levels; this is correlated with a decrease in symptoms.

6.2 Dust mite preparations

10 Whole dust mite preparation from *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* (DMA) including mite bodies, feces and culture medium (10 gm) is added to phosphate buffered saline pH 7.3, (10 ml/gm) and stirred overnight at 4° C. The resultant mixture is centrifuged
15 at 100,000 g for 1 hour. Supernatant is removed and precipitated with 50% saturated ammonium sulfate. The sediment is removed by centrifugation at 4,000 rpm for 15 minutes, and resuspended in phosphate buffered saline pH 7.3 (PBS). This preparation is dialyzed against PBS (4
20 changes of 4 litres over 48 hours), and the product stored at -20° C (DMA). Its composition is characterized by SDS PAGE analysis. The concentrations of *Der p I* protein is determined by immunoassay, compared to standards provided by Dr. Martin Chapman, University of
25 Virginia, Charlottesville, USA (Luczinska et al., 1989, J. Immunol. Methods, 118:227-2359).

6.2.1 Purified Group I Mite Allergens

Der p I and *Der f I* preparations are isolated from
30 DMA extracts of the appropriate mites by affinity chromatography on insolubilized anti-*Der II* monoclonal antibody (for example, 1114/2F10 - Table 1: 7AI, Lombardero et al, 1990, J. Immunol., 144:1353-1360, 1990. In an example, murine monoclonal antibody 1114/2F10 (50
35 mg) is coupled to cyanogen bromide activated Sepharose 4B. Dust mite extract is applied to the column

containing 1114/2F10 mAb-Sepharose 4B and non-bound material eluted by extensive washing with PBS. The bound product is then eluted with 0.005 M glycine in 50% ethylene glycol pH 10. the eluted product is precipitated
5 with 50% ammonium sulphate, re-dissolved in PBS and dialysed against several changes of 2L PBS. The final product is stored at -70° C.

6.2.2 Purification of Group II Mite Allergens

10

Der p II and *Der f II* preparations are isolated, from DMA extracts of the appropriate mites by affinity chromatography on insolubilized anti-*Der II* monoclonal antibody (for example, 1114/2F10 - Table 1: 7AI; Lombardero et al., 1990, J. Immunol., 144:1353-1360). In
15 an example, murine monoclonal antibody 1114/2F10 (50 mg) is coupled to cyanogen bromide activated Sepharose 4B. Dust mite extract is applied to the column containing 1114/2F10 LUB-Sepharose 4B and nonbound material eluted
20 by extensive washing with PBS. The bound product is then eluted with 0.005M glycine in 50% ethylene glycol pH 10. The eluted product is precipitated with 50% ammonium sulphate, re-dissolved in PBS and dialysed against several changes of 2L PBS. The final product is stored
25 at -70° C.

6.3 Production of murine anti-*Der p* and anti-*Der f* monoclonal antibodies

6.3.1 Immunization protocol

30

BALB/c mice are immunized according to the following schedule: 25 µg DMA in Freund's complete adjuvant (FCA), delivered intraperitoneally, as the initial immunization (Day 0) then, at various intervals, between days 9 and 56, they are again injected with 25 µg DMA in incomplete
35 Freund's adjuvant (IFA), intraperitoneally, two to four times. Usually 3 days prior to sacrifice 25 µg of DMA in

PBS is injected intravenously. Mice are killed on days 45 to 60, and single cell suspensions of the spleen are prepared and fused with mouse myeloma NSO cells by established procedures known to those skilled in the art (Embleton et al., Br. J. Cancer, 43:582-587, 1981; Kohler and Milstein, Nature, 256:495-497, 1975; US Patent No. 4,376,110).

6.3.2 Hybridoma selection protocol

Hybridomas are selected by screening of culture supernatants for antibody reacting with a PBS extract of dust mite preparations (DMA) by an ELISA test (ELISA and Other Solid Phase Immunoassays Eds., D.M. Kemeny and S.J. Challacombe, 1988) (Wiley). Briefly, polyvinyl microtiter plates (Falcon "MICROTEST III" or the like) are coated with the equivalent of 5 μ g/well Der p I by overnight incubation of a dust mite preparation (DMA). To reduce non-specific antibody binding due to uncoated reactive sites in wells, the microtiter plates are blocked with casein in tris buffer pH 7.3. Tissue culture supernatant is added to the microtiter plate wells for 45 minutes, then excess removed by washing in PBS. Peroxidase conjugated rabbit antibody to mouse immunoglobulin is added and bound antibody detected by color formation following addition of substrate 2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid) (ABTS). Hybridomas selected in the primary screen are cloned two times and hybridoma lines established. Monoclonal antibodies (mAbs) from cloned hybridomas are purified by various procedures and further characterized. Immunoblotting with mAb purified from hybridoma supernatants is carried out in which Der p extract is subjected to SDS-PAGE followed by transfer to nitrocellulose and subsequent staining with mAb (Thompson et al., 1988, J. Immunol., 64:311-314. By comparison with standard (eg, Der p I) this allows components of the

Der p extract reacting with the mAb to be identified. The characteristics of selected mAbs in comparison with other anti-dust mite antibodies is further established using a blocking assay. In this procedure the mAb under
5 investigation is added to microtiter plates coated with dust mite extract (DMA) so blocking antigen sites. Inhibition of the binding of defined antibodies such as anti-*Der p I* antibody 5H8 (Chapman et al., J. Immunol., 139:1479-1484, 1987) indicates that the mAb under test
10 recognizes the same or closely related epitopes on DMA to the standard antibody. To measure inhibition of mAb binding, biotinylated mAb is added to the blocked microtiter plates and bound antibody is detected by addition of streptavidin-peroxidase conjugates, followed
15 after washing by the substrate (ABTS).

The ability of murine mAbs to inhibit binding of antibodies in serum of patients sensitized to dust mite allergens is similarly detected by a blocking assay. In these tests, the mAb under investigation is added to
20 microtiter plates coated with dust mite extract. Inhibition of the binding of patients serum is then detected by first adding patients serum to microtiter plates, washing and detection of bound human Ig with goat-antihuman Ig peroxidase conjugate followed by
25 substrate (ABTS).

6.3.3 Anti-idiotypic antibody response to murine anti-*Der p* and anti-*Der f* monoclonal antibodies

Murine anti-idiotypic antibodies to monoclonal
30 antibodies are produced by immunizing inbred BALB/c mice with monoclonal antibodies coupled to keyhole limpet hemocyanin together with Freund's complete, then incomplete, adjuvant. In an example, BALB/c mice are
immunized first with mAb-KLH (50 µg) incomplete Freund's
35 adjuvant and then twice at two week intervals with mAb-KLH (50 µg) in incomplete adjuvant. Antisera are then

obtained 7 to 14 days later as a source of antibodies used to define the idiotypes of anti-Der *p I* monoclonal antibodies 2C7 (Table 2) and 4C1 (Chapman et al., 1987, J. Immunol., 139:1479-1484). Binding of mAb 2C7 to Der *p I* is detected by radioimmunoassay using ¹²⁵I labeled mAb 2C7. Inhibition of this binding by a range of anti-idiotypic antibodies is detected by mixing ¹²⁵I labeled mAb 2C7 with antiserum before addition to microliter plates coated with Der *p* allergen. Binding of 2C7 is inhibited only by anti-idiotypic antibody to 2C7. Binding of 4C1 to Der *p I* is only inhibited by anti-idiotypic antibody to 4C1. These findings show that although mAbs 2C7 and 4C1 recognize closely related epitopes on Der *p I* they possess separate and distinct idiotypes.

15

6.4 Murine anti-Der *p* hybridoma cell lines

6.4.1 mAb 1102/H11

The mAb is obtained from culture supernatants of hybridoma 1102/H11 cells maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. mAb 1102/H11 is purified by affinity chromatography on Sepharose protein G (Pharmacia) and isotype analysis established the antibody to be immunoglobulin IgG₁.

The reactivity of 1102/H11 with dust mite-associated antigen has been established by ELISA assay of its binding to a DMA preparation. Further characterization by immunoblotting against Der *p* proteins after SDS PAGE and transfer to nitrocellulose indicated mAb 1102/H11 reacts with a 24 kDa component equivalent to that detected murine mAb 5H8 (clone 5H8 C12 D9) which binds to an epitope on Der *p I* (Chapman et al., J. Immunol., 139:1479-1484, 1987).

The blocking assay was used to further investigate the relationships between dust mite antigen epitopes reacting with mAbs 1102/H11 and 4C1. This is illustrated in Figure 4 which shows that mAb 1102/H11 inhibited

binding of biotinylated 4C1 to dust mite extract (DMA). These findings confirm that mAb H11 reacts with a *Der p* epitope identical to, or closely related to, the epitope recognized by mAb 4C1 (Chapman et al., J. Immunol., 139:1479-1484, 1987).

Table 1 lists human monoclonal antibodies produced by hybridomas formed by fusion of human lymphocytes from tonsils or peripheral blood with a human-mouse heteromyeloma EL 41.

TABLE 1

Human Anti-dust Mite Monoclonal Antibodies

ELISA OD on Plates Coated with

	Hybridoma	Lymphocyte	DMA ²	Casein Reactivity		
15	with:	Donor ¹				
					Der p I	Der p II ³
	DM27	1E11	0.196	0.049	+	-
	DM27	1A11 Tonsil	0.065	0.000	-	+
	DM27	1F10	0.094	0.000	-	+
20	DM27	2G9	0.111	0.000	+	-
	DM27	1D11	0.160	0.019	+	-
	DM28	2A2 PBL	0.172	0.043	-	+
	DM31	2E9	0.391	0.176	+	-
	DM31	1H2	0.138	0.072	-	+
25	DM31	1G7 Tonsil	0.179	0.096	+	-
	DM31	2B2	0.191	0.000	+	-
	DM31	2C10	0.202	0.000	+	-

6.4.2 mAb 1107/2B11

Antibody is obtained from culture supernatants of hybridoma 1107/2B11 which is maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. mAb 1107/2B11 is purified by affinity chromatography on Sepharose protein G (Pharmacia) and isotype characterized to establish the antibody to be added. Characterization

of the dust mite antigen reactivity by immunoblotting after SDS PAGE and transfer to nitrocellulose indicated that it reacts with a protein with a molecular weight of 27 kDa.

5

6.4.3 Other murine anti-dust mite mAb

Similar procedures have been used to produce a range of murine mAb reacting with dust mite *Der p* and *Der f* antigens (Table 2).

10

TABLE 2

	mAb	Isotype	Allergen	Mw
	1102/H11	IgG ₁	<i>Der p I</i>	24000
15	1107/2B11	IgG ₁	<i>Der p I</i>	27000
	1111/A2	IgG ₁	<i>Der p I</i>	24000
	1114/1F7	IgG _{2a}	<i>Der p I</i>	24000
	1114/2F10	IgG _{2a}	<i>Der p II</i>	15000
20	1119/IC12	IgM	ND	27000

6.5 Selection of murine anti-*Der p* mAb for down-regulating immune responses to *Der p I* and *Der p II* allergens

An additional series of anti-dust mite monoclonal antibodies have been selected which are capable of down-regulating the allergic response to dust mite. These monoclonal antibodies are directed against *Der p I* and *Der p II*, the two major allergens in dust mite (Table 2). Those monoclonal anti-*Der p I* and *Der p II* antibodies also cross-react with *Der f I* and *Der f II*, and so are useful for regulating allergic responses to allergies in either *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae*.

35

The selected mAbs also recognize immunodominant regions of the allergens as determined by their capacity

to block interaction of serum IgE antibodies to either *Der p I* or *Der p II*. Data obtained using mAb 2C7 directed against *Der p I* provide an example of antibody selection, characterization and assessment.

5

6.5.1 Anti-*Der p I* mAb 2C7

Anti-*Der p I* mAb 2C7 was produced as described in section 6.3, and purified from hybridoma culture supernatants by Sepharose-protein A affinity chromatography. This mAb (isotype IgG_{2b}) reacts with *Der p I* and *Der f I* (Table 3) as demonstrated by ELISA assay (described in section 6.3.2).

In previous studies reported by Chapman and associates (Chapman et al., J. Immunol., 139:1479-1484, 1987), murine mAb 4C1 was shown to react with an immunodominant epitope on *Der p I* since it blocked up to 40% of the binding of serum IgE to *Der p I*. Also, mAb 4C1 is cross-reactive with epitopes on *Der p I* and *Der f I*. The close relationship of the *Der p I* epitope reacting with mAb 2C7 and 4C1 was demonstrated using cross-blocking assays. In this procedure ELISA microtiter plates coated with dust mite extracts are incubated with a range of concentrations of mAb 2C7. After washing, binding of mAb 4C1 is determined using biotin-labeled mAb 4C1. In this assay biotinylated mAb 4C1 is detected using streptavidin-conjugated horseradish peroxidase. Using this inhibition assay, it was shown that mAb 2C7 completely blocked the antigen sites on *Der p I* which react with mAb 4C1 (Figure 9). These findings indicate that the epitopes on *Der p I* recognized by mAbs 2C7 and 4C1 are identical or closely related. In comparison, anti-*Der p I* mAb H11 (Table 3) was unable to block the binding of biotinylated mAb 4C1 to *Der p I*. mAb H11 therefore recognizes a *Der p I* epitope unrelated to that reacting with mAb 4C1.

6.5.2 mAb idiotype characterization

To further explore the relationship of mAb 2C7 and 4C1 binding epitopes, the idiotypes of these two mAbs were defined. Polyclonal anti-idiotypic

5

TABLE 3

Reactivity of Anti-Dust Mite Monoclonal Antibodies

10 Reactivity (ELISA OD 405 nm) with:

Monoclonal Antibody		<i>Der p I</i>	<i>Der f I</i>	Control ¹
2C7		0.56	0.44	0.05
4C1*		0.60	0.33	0.04
H11		0.43	0.39	0.04
15 337**		0.07	0.05	0.02
		<i>Der p II</i>		
2H5		0.70		0.09
6D6*		0.60		0.07
337**		0.09		0.04

20

¹ ELISA plate wells uncoated

(*) mAb provided by Dr. M. Chapman, Division of Allergy and Clinical Immunology, University of Virginia, Charlottesville, Virginia, USA

25 (***) Control mAb anti-carcinoembryonic antigen provided by Prof. R.W. Baldwin, Cancer Research Campaign Laboratories, University of Nottingham, Nottingham, UK

antibodies were produced to a range of murine anti-*Der p I* monoclonal antibodies (4C1, 2C7, 5H8), anti-*Der p II* mAb (6D6) and control mAb 337 (anti-carcinoembryonic antigen, CEA). These polyclonal anti-idiotypic antisera were produced by immunizing BALB/c mice with mAbs conjugated to keyhole limpet hemocyanin (KLH) in complete and then incomplete Freund's adjuvant.

35

Anti-idiotypic anti-sera were assayed for the capacity to inhibit binding of anti *Der p I* mAbs (2C7, 4C1, and 10B9) to *Der p I*. In this assay various dilutions of polyclonal anti-idiotypic anti-sera were
5 mixed and incubated with ¹²⁵I labeled murine monoclonal antibody so as to allow reaction with the mAb. The mixture was then added to ELISA microtiter plates coated with *Der p I* and reaction determined by radioimmunoassay. As shown in Figure 10, anti-mAb 2C7 serum completely
10 inhibited binding of 2C7 to *Der p I*. In contrast anti-4C1 serum did not produce any inhibition of 2C7 binding to *Der p I*. These findings indicate that mAbs 2C7 and 4C1 recognize closely related but non-identical *Der p I* epitopes.

15

6.5.3 Relationship between *Der p I* epitopes inducing human IgE antibodies and those reacting with murine Anti-*Der p I* mAbs

In selecting murine mAbs for down-regulating human
20 IgE reactivity to *Der p I* (and *Der p II*) a useful criterion is that both antibody species react with the same allergen epitope. Also the dust mite allergen epitope preferably should be an immunodominant component accounting for a major component i.e., 25% or greater,
25 preferably at least 40%, 50%, or 60% of the human IgE response. For greater general clinical utility as a therapeutic, a preferred monoclonal antibody should exhibit greater than 40% inhibition of IgE binding to the allergen in a majority of human subjects. This has been
30 demonstrated with a range of anti-*Der p I* mAb by experiments showing that they block up to 80% of the binding of serum IgE antibody to *Der p I* (Figure 11).

In these tests, serum from 6 atopic dermatitis subjects with high levels of serum IgE antibodies to *Der p I* were examined. Serum samples, diluted to a level
35 giving sub-saturating amounts of IgE antibody binding to

Der p I coated ELISA microtiter plates, were mixed with murine mAb (100 µg). This mixture was added to Der p I-coated microtiter plates. After incubation overnight at 4° C bound human IgE was detected by ELISA using goat anti-human IgE horseradish peroxidase conjugate. As shown in Figure 11, mAb 2C7 was the most effective of the four anti-Der p I mAbs in inhibiting binding of human serum IgE to Der p I. With this mAb, at least 50% of serum IgE binding was obtained with 5 of 6 patient serum samples tested. The maximum inhibition obtained was 80%, with serum from patient A368. In comparison, greater than 40% inhibition of serum IgE binding was only obtained with 1/6 serum samples using murine mAb 4C1 (Chapman et al., J. Immunol., 139:1479-1484, 1987).

6.5.4 Identification of idiotypes of mAb 2C7 and Human anti-Der p I IgE Antibody

In selecting monoclonal antibodies to dust mite allergens for therapy, a significant criterion is that the idiotypic of the selected mAb is identical, or closely related, to the idiotypic of human IgE antibodies. To establish this with mAb 2C7, IgE antibodies from serum of atopic dermatitis patients were captured on ELISA microtiter plates coated with goat anti-human IgE antiserum as described in the protocol *infra*. The reactivity of bound human IgE was then determined with a range of polyclonal anti-idiotypic antibodies produced against murine anti-Der p I mAbs. As summarized in Table 4, polyclonal mouse anti-idiotypic antibodies to mAb 2C7 reacted with all six IgE preparations from serum of atopic dermatitis patients with high anti-Der p I antibody levels. In comparison, none of the IgE samples bound to anti-idiotypic antisera raised against mAb 4C1. These observations indicate that the idiotypic of mAb 2C7 is closely related to that of human IgE. This supports the algorithm for choice of a therapeutic antibody, which

indicates that stimulation of anti-idiotypic immune responses to mAb 2C7 is appropriate for immunoregulation of *Der p I* allergy.

5

TABLE 4

Binding of Anti-Idiotypic Antibodies
Produced Against Mouse Anti-*Der p I*
Monoclonal Antibodies to Serum IgE
from Atopic Dermatitis Patients*

10 Binding (ELISA, OD 405 nm) of anti-idiotypic antibody to:

	Serum IgE from Patient	mAb 4C1	mAb 2C7	Normal Mouse Serum
	1	0	1.03	0
15	2	0.1	1.03	0
	3	0.04	1.0	0
	4	0.02	1.0	0
	5	0.03	1.08	0
	6	0.05	0.87	-0.1
20	7	0.04	0.72	0

* Microtiter plates are coated with goat anti-human IgE, and patient sera are added to allow capture of anti-*Der p I* IgE. Binding of polyclonal mouse antibody produced against murine mAb determined by ELISA (OD 405 nm)

25

6.6 Chimerization-humanization of murine monoclonal antibodies

Monoclonal antibodies of murine origin are humanized by various methods so as to reduce the immunogenicity of the proteins with respect to the murine immunoglobulin component. One way involves production of chimeric antibody by linking cDNA molecule encoding the V region of the murine mAb to DNA encoding the human constant region. This can be effected using several approaches described in patents (Cabilly et al., US Patent 4,816,567 (28 March, 1989); Taniguchi et al., Eur. Patent EP 171496

35

(19 February 1986) and Kudo et al., Eur. Patent EP 184187 (11 June, 1986)) and publications including Cabilly et al. Proc. Natl. Acad. Sci., USA, 81:3273-3277, 1984; Morrison et al., Proc. Natl. Acad. Sci., USA, 81:6851-6855, 1984; Neuberger et al., Nature, 314:268-270, 1985; Tan et al., J. Immunol., 135:3564-3567, 1985; Sun et al., Proc. Natl. Acad. Sci., USA, 84:214-218, 1987; Lui et al., J. Immunol., 139:3521-3526, 1987 and Horowitz, A.H. Proc. Natl. Acad. Sci., USA, 95:8676-8682, 1988. The chimerization procedure involves:

- (1) Isolation of messenger RNA (mRNA) from mouse hybridoma cells, then cloning and cDNA production.
- (2) Preparation of a full length cDNA library from purified mRNA from which appropriate variable (V) region gene fragments of the light (L) and heavy (H) chain genes can be identified, sequenced and made compatible with a constant (C) region gene segment.
- (3) Preparation of C region gene segment modules by cDNA preparation and cloning.
- (4) Construction of complete H or L chain coding sequences by linkage of the cloned specific immunoglobulin V region gene segments to cloned human C region segment modules.
- (5) Expression and production of chimeric L and H chains in prokaryotic and eukaryotic cells.

6.7 CDR grafting

Humanized monoclonal antibodies containing only murine variable regions and constructed by CDR grafting/reshaping using various techniques (Winters and Milstein, Nature, 349:293-299, 1991; Riechmann et al.,

Nature, 332:323-327, 1988-, Hird et al., Br. J. Cancer, 64:911-914, 1991; Gussow and Seemann, Methods in Enzymology, 203:99-121, 1991). The complementary determining regions (CDRs) of a murine mAb are

5 transplanted onto the corresponding regions in a human antibody. The CDRs (three in antibody heavy chains and three in light chains) are the regions of the murine mAb which bind to the antigen epitope. Transplantation of the CDRs is achieved by manipulation whereby CDR DNA

10 sequences are determined by cloning of murine heavy and light chain variable (V) region gene segments and transfer to corresponding human V regions by site-directed mutagenesis. Human constant region gene segments of the desired isotype are added and the

15 humanized heavy and light chain genes are co-expressed in mammalian cells to produce humanized antibodies.

A general procedure has been developed to graft CDRs from rodent monoclonal antibodies onto human FRs to make fully humanized antibodies or antibody fragments using

20 PCR methodology (A. P. Lewis and J. S. Crowe, Gene, 1991, 297-302). PCR primers which bind to the FR regions bracketing the CDRs have been developed; these permit amplification of the CDR from hybridoma cells secreting the mAb of interest. The PCR product is cloned and

25 sequenced, and joined to the framework and constant regions of choice.

7. Example: production of human anti-Der p monoclonal antibodies

30 Human monoclonal antibodies reacting with Der p antigens are produced by various procedures. These include immortalization of antibody-producing cells from human donors sensitive to DMA by construction of human hybridomas or human-mouse heterohybridomas using

35 techniques well known to those versed in the art (Austin et al., Immunology, 67:525-530, 1989) or by Epstein Barr

virus transformation (Roder et al., Meth. in Enzymology, 121:140-167, 1986). Human antibodies are also obtained by repertoire cloning (Marks et al., J. Mol. Biol., 222:581-597, 1991; Persson et al., Proc. Natl. Acad. Sci., 86:2432-2436, 1991; Huse et al., Science, 246:1275-1281, 1989; Kang et al., Proc. Natl. Acad. Sci. USA, 80:4363-4366; Duchosal et al., Nature, 355:258-262, 1992).

10 7.1 Hybridoma Production

Lymphocytes are obtained from various tissues including peripheral blood, tonsils and spleen from human subjects sensitized to dust mite. Cell preparations are obtained by "FICOLL-HYPAQUE" density centrifugation and
15 fused immediately after separation or following *in vitro* stimulation. Various *in vitro* stimulation methods are used including treatment of lymphocytes cultured in medium RPMI 1640 with dust mite extracts. In some cases *in vitro* culture conditions include immunological
20 adjuvants such as adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, Sigma).

7.1.1 Fusion Protocol

Mouse/human heteromyeloma EL41 cells (deposited on
25 May 16, 1990 with the European Collection of Animal Cell Cultures and having Accession No. 90051602) as described by Austin et al., Immunology, 1989, 67:525-530, are fused with human lymphocytes reactive with dust mite antigen using polyethylene glycol according to the method of
30 Galfre et al., Nature, 1977, 266:550. For example, lymphocytes are placed in 30 ml plastic universal bottles and washed twice with growth medium such as RPMI 1640 and the number of lymphocytes determined by hemocytometer counting. EL41 cells are harvested, washed and counted
35 in a similar fashion. The two cell populations are then mixed at a lymphocyte:fusion partner ratio of 2:1 and

washed for a third time. Following centrifugation the medium is discarded and 0.8 ml of 50% PEG pipetted over a 1 minute period onto the cell pellet with gentle agitation. The mixture is left for one minute then 1.0 ml of medium added over a further 1 minute period followed by the addition of 20 ml of medium added slowly over a 5 minute period. The cells are then centrifuged at 1200 rpm for 5 minutes, the medium discarded, and the cells resuspended by gentle pipetting in a 2 ml pipette. Twenty ml of selection medium (RPMI 1640 + 10%FCS + 10^{-4} M hypoxanthine, 10^{-5} M methotrexate and 1.6×10^{-6} M thymidine) is finally added to the cell suspension and the cells aliquoted into two 96 well tissue culture microtiter plates containing mt peritoneal exudate cells (2.5×10^3 / well) as a feeder layer. An additional 100 μ l of media is also added to each well. The selection medium is changed every 72 hours, until resulting hybridomas have reached 50% confluence, then every 48 hours. The growth of the hybridomas is regularly checked and when the colonies have covered 50% of the well the supernatant is analyzed for Ig reactive with dust mite antigen. Hybridomas producing Ig of interest are immediately cloned. Established hybridomas are frozen down in aliquots of $2-5 \times 10^6$ cells in 1 ml of 95% FCS and 5% Dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

7.1.2 Lymphocyte Preparation

Peripheral blood lymphocytes are prepared by diluting heparinized blood 1:2 v/v with balanced salt solution and centrifugation over lymphocyte separation medium (Flow Laboratories, Irvine, Scotland).

Lymphocytes are prepared from tonsil or spleen by teasing chopped tissue through a 120 gauge grid into balanced salt solution. After washing, the lymphocytes

are counted, viability being assessed by Trypan blue dye exclusion.

7.1.3 *in vitro* stimulation

- 5 i. Adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, Boss, Brain Res., 1984, 291:193) and dust mite preparation.

10 Lymphocytes (2×10^6 /ml) are cultured up to 5 days with adjuvant peptide ($10 \mu\text{g/ml}$) and dust mite antigen ($5 \mu\text{g/ml}$) in medium RPMI 1640 containing 10% fetal calf serum. The lymphocytes are then fused immediately with the fusion partner heteromyeloma EL41 (Austin et al., Immunology, 1989, 67:525-530).

- 15 ii. Pretreatment with L-Leucyl-L-leucine-methyl ester.

20 Treatment of lymphocyte preparations with L-Leucyl-L-leucine-methyl ester eliminates large granular lymphocytes, macrophages, C7L precursors and effectors, some CD8+ suppressor T cells and NK cells. It is used to enhance the *in vitro* immunization procedure for the production of human monoclonal antibodies (Borrebaeck et al., 1988, Proc. Natl. Acad. Sci. USA., 85:3995). Briefly, lymphocytes are cultured for 15 mins at room temperature in medium RPMI 1640 containing $250 \mu\text{M}$ L-Leucyl-L-leucine-methyl ester. The cells are then washed and cultured for 2-8 days in medium RPMI 1640 supplemented with 10% human AB serum, $50 \mu\text{M}$ 2-mercaptoethanol, 5IU recombinant interleukin 2 (rIL-2), 25% v/v supernatant from irradiated human T cells stimulated with pokeweed mitogen (PWM) (24 hr culture) and dust mite antigen 5-100 ng/ml. Recombinant IL-4 (50U) and rIL-6 (100U) is also added to some cultures as an extra lymphokine boost.

35 Following stimulation lymphocytes are fused with the heteromyeloma EL41.

iii. *in vitro* primed splenocytes (Boerner et al., 1991, J. Immunol., 147:86-95).

Splenocytes from two donors are mixed at equal concentrations and cultured 3×10^6 /ml in RPMI 1640 medium containing 10% FCS and dust mite antigen 1-10 μ g/ml for 2-5 days. The lymphocytes are then fused with the fusion partner heteromyeloma EL41.

7.2 Hybridoma Selection

Hybridomas are selected by screening of culture supernatants for antibody reacting with dust mite antigen (DMA) preparations (see section 6.3.2) using ELISA. Briefly polyvinyl microtiter plates (Falcon "MICROTEST III" or the like) are coated with dust mite preparation containing the equivalent of 0.5 μ g/well *Der p I*. To reduce non-specific antibody binding to uncoated surface sites in wells, microtiter plates are blocked with casein in tris-saline buffer pH 7.3. Hybridoma supernatant is added to the wells for 45 minutes, then the excess removed by washing in PBS. Peroxidase conjugated rabbit antibody to human immunoglobulin is added and bound antibody detected by color formation following addition of substrate (ABTS). Hybridomas selected in the primary screen are cloned two times and hybridoma lines established using culture procedures for stable growth. Hybridoma cell preparations of the initial fusion product and cloned lines are preserved by freezing and storing in liquid nitrogen.

Monoclonal antibodies are purified by various procedures and are characterized. Immunoblotting is carried out in which *Der p* and *Der f* extracts are subjected to SDS PAGE followed by transfer to nitrocellulose and subsequent staining with mAb (Thompson et al., Immunol., 64:311-314, 1988; Chapman et al., J. Immunol., 139:1479-1484, 1987; Platts-Mills and Chapman, J. Allergy Clin. Immunol., 80:755-775, 1987) so as to

define the molecular weights of the dust mite allergens bound by each mAb.

The relationship of human mAb to antibodies produced in human subjects sensitized to dust mite allergens is detected using several immunoassays well known to those versed in the art (as an example Didierlaurent and Garcelon J. Immunol. Meth., 145:221,41, 1991). In one approach microtiter plates (Chapman et al., J. Immunol., 139:1479-1484, 1987) or cyanogenbromide activated paper discs (Didierlaurent and Garcelon, J. Immunol. Meth., 145:33-41, 1991) are coated with dust mite extract. Serum from allergic donors is incubated in the microtiter wells or discs and after washing inhibition of the reactivity with the human mAb detected. For this purpose, mAb is conjugated to biotin by well established methods. The inhibition of binding of biotinylated mAb in microtiter wells or discs is then determined by incubation first with streptavidin-biotin-peroxidase conjugate (Amersham, UK) and then with o-phenylenediamine (OPD).

7.3 Epstein-Barr Virus Transformation

Epstein Barr virus (EBV) is a herpes virus which selectively infects human B lymphocytes expressing the CR2 complement receptor. Infection *in vitro* of B lymphocytes leads to permanent stimulation of cell growth, with the preservation of B cell characteristics such as immunoglobulin secretion (Roder et al., Methods in Enzymology, 121:140-174, 1986). For EBV transformation tissue culture supernatant obtained from the marmoset cell line B95-8 is used. This cell line, when cultured in Standard RPMI 1640 medium with 10% FCS, releases large quantities of virus.

Tissue culture supernatant is taken from B95-8 cells grown to confluence, this is then used at a dilution of 1/2 to 1/10 in RPMI 1640 with 10% FCS to infect human

lymphocytes ($2 \times 10^6/\text{ml}$). The cells are incubated for 2 hours at 37°C then washed and cultured in RPMI 1640 with 10% FCS or cultured without washing for 24-48 hours after which time a 50% medium change is performed every 3-4 days.

The cells are cultured in "COSTAR" plates (2×10^6 /well) or U bottom microtiter plates (10^4 /Well). Irradiated mouse spleen cells or human peripheral blood mononuclear cells can be used as a feeder layer.

7.4 Repertoire cloning

Antibodies reacting with *Der p* and *Der f* allergens were produced by procedures based upon antigen selection from a combinatorial library and cloned (Orlandi et al., Proc. Nat. Acad. Sci., 86:3833-3837, 1989; Persson et al., Proc. Nat. Acad. Sci. USA, 88:2432-24360, 1991; Kang et al., Proc. Nat. Acad. Sci. USA, 88:4363-4366; Duchosal et al., Nature, 355:258-262, 1992; Clackson et al., Nature, 352:624-628, 1991; Marks et al., J. Mol. Biol., 222:581-597, 1991). The general approaches well known to those versed in the art include extraction of messenger RNA (mRNA) from human lymphocytes derived from various tissues including peripheral blood (PBL), tonsillar tissue (Ton-Ly) and spleen (Spl-Ly). The lymphocytes may be derived from human allergic subjects and in addition may be initially stimulated by exposure to allergens and immunological adjuvants as already described. Another procedure for stimulating lymphocytes involved injecting PBL, Ton-Ly etc. into severe combined immune deficiency (SCID) mice and stimulation of injected mice with allergen preparations such as the whole dust mite extracts (DMA) or purified proteins such as *Der p I* and *Der p II*. Mice may also be treated with immunological adjuvants such as bacillus Calmette Guerin (BCG) or by combining immunogen preparations (*Der p I/II* etc) with

aluminum hydroxide gel, variously described as adjuvant alum.

The polymerase chain reaction (PCR) together with appropriate primers is used to amplify the variable heavy (VH) and variable light (VL) genes from the mRNA preparations using well established procedures (Orlandi et al., Proc. Nat. Acad. Sci. USA, 86:3833-3837, 1989 as an example). The RNA preparations are used to construct Fab combinatorial libraries in phage (Persson et al., Proc. Nat. Acad. Sci., 88:2432-2436, 1991; Huse et al., Science, 246:1275-1281, 1989). Clones selected by screening are plaque-purified and the phagemids excised and used to transform *Escherichia coli*. Fabs from culture transformants were then produced by procedures well known to those versed in the art (Gussow and Seemann, Methods in Enzymology, 99:121, 1991, Pluckthun, Biotechnology, 9:545-551, 1991, Nature, 347:497-498, 1991).

7.5 Results

7.5.1 DM27 1E11

Human tonsillar lymphocytes (2×10^6 cells) were stimulated for three days in medium RPMI 1640 together with adjuvant peptide (Sigma) 10 μ g and dust mite extract (5 μ g). Cell preparations were collected, resuspended in RPMI for fusion with human-mouse heteromyeloma EL41. From this fusion, human hybridoma DM27/1E11 was selected. The hybridoma was cloned two times and growth maintained in medium RPMI 1640 supplemented with 10% fetal calf serum. Human mAb DM27/1E11 was obtained from hybridoma supernatant by affinity chromatography on Sepharose protein G (Pharmacia). Antibody DM27/1E1 reacted with dust mite extract and with purified *Der p I* preparations (Table 5). Isotype characterization of the antibody established it to be IgG₁.

TABLE 5
Human anti-dust mite monoclonal antibodies

5	ELISA OD on Plates					
	Coated with:					
	Hybridoma	Lymphocyte Donor ¹	DMA ²	Casein	Reactivity with:	
					<i>Der p I</i>	<i>Der p II</i> ³
10	DM27 1E11		0.196	0.049	+	-
	DM27 1A11	Tonsil	0.065	0.000	-	+
	DM27 1F10		0.094	0.000	-	+
	DM27 2G9		0.111	0.000	+	-
	DM27 1D11		0.160	0.019	+	-
	DM28 2A2	PBL	0.172	0.043	-	+
15	DM31 2E9		0.391	0.176	+	-
	DM31 1H2		0.138	0.072	-	+
	DM31 1G7	Tonsil	0.179	0.096	+	-
	DM31 2B2		0.191	0.000	+	-
	DM31 2C10		0.202	0.000	+	-
20	¹	Fused with human-mouse heteromyeloma				
	²	Dust mite extract				
	³	ELISA test				

7.5.2 Other human anti-dust mite Mabs

25 Table 6 lists representative human anti-dust mite allergen monoclonal antibodies generated by fusion of human lymphocytes with human-mouse heteromyeloma ELAI. Lymphocytes were obtained from human peripheral blood (hybridomas DM28/1D11, DM28/2B2, DM28/2E9) and from
30 tonsillar tissue (DM27/1E11 to 2G9 and DM31/1H2 to 2C10).

TABLE 6

Immunoreactivity of Human Antibodies From EBV--
Transformed Lymphocyte Lines

5	EBV -	Tonsillar	Antibody Reactivity		
	Transformed	Tissue ²	DMA	Der p I	Der p II
	Tonsillar Lymphocyte ³				
10	EVA-11	T10	+	+	-
	EBV-12	T14	+	-	+
	EBV-13 A	T13	+	+	-
	B	T13	+	+	+
	C	T13	+	-	-
15	D	T13	+	+	-
	EBV-14 A	T14	+	-	+
	B	T14	+	+	-

¹ Binding of antibody determined by ELISA assay with dust mite extract and purified proteins

20 ² Tonsillar tissue obtained at tonsillectomy from allergic donors

³ Tonsillar lymphocytes transformed with Epstein Barr Virus (EBV)

25 The mAbs reacted in ELISA tests with dust mite extracts as compared to control microtiter wells coated only with casein. Specificity tests indicated that 9/11 reacted with *Der p I*.

30 7.5.3 *in vivo* Testing of Anti-Der p Mab for Down-Regulating T Lymphocyte Mediated Responses to Dust Mite Allergens

The importance of T lymphocyte responses in the pathogenesis of allergic conditions resulting from exposure to dust mite allergens is clearly established (Ishizaka, Ann. Rev. Immunol., 2:159-182, 1984; O'Hehir et al., Int. Allergy Appl. Immunol., 88:170-172, 1989;

Frew and Kay, J. Immunol., 141:4158, 1988; Alexander et al., Lancet, 339:324-328, 1992).

5 The synthesis of specific IgE antibody and the differentiation and growth of effector cells such as mast cells and eosinophils are dependent upon the activation of CD4+ cells (O'Hehir et al.,).

Control of T lymphocyte responses to dust mite allergens is an important pathway in treating allergic diseases caused by these substances. This can be
10 effected by treatment with immunosuppressive drugs. In view of the overall toxicities of agents such as cyclosporin A these agents are not considered applicable for the treatment of most asthma patients. Alternative approaches for suppressing T cell responses by
15 manipulating the immune network are more appropriate since these treatments are much less toxic.

From the above considerations evaluation of the potential of anti-dust mite mAbs for controlling allergic diseases is evaluated initially by demonstrating that
20 treatment suppresses T lymphocyte mediated responses.

8. Inhibition of Delayed Type Hypersensitivity (DTH) Responses in Mice to Dust Mite Allergens

25 8.1 Induction of DTH in Mice to Dust Mite Extracts

Dust mite extract (DMA) 10 to 200 μ g mixed with 1:1 v/v with complete Freund's adjuvant (CFA) is injected subcutaneously into two sites in BALB/c mice. Control mice are injected with CFA mixed with PBS or an
30 irrelevant protein. Mice are challenged 5 to 28 days later by injection of DMA (10 μ g) or purified *Der p I/II* preparations given intradermally into the left ear pinna using the procedures described by Austin et al., 1991 (Austin et al., J. Natl. Cancer Inst., 1991). The
35 uninjected right ear serves as the control. Twenty four and 48 hours after challenge, the thickness of the ears

is measured. The differences between challenged and non-challenged ears is calculated and the measurements expressed as increment in ear swelling as a measure of the DTH response. (Austin et al., J. Nat. Cancer Inst., 1991, Kuriboyashi et al., Cell Immunol., 108:366-377, 1987, Morikawa et al., Immunology, 74:146-152, 1991.

To measure the effect of anti-dust mite mAb treatment, mice receive antibody at various times before or after sensitization with DNU. mAb may be administered by various routes and immunological adjuvants may be included in the preparation.

Suppression of DTH responses against dust mites may be improved by combination treatment with mAbs reacting with different epitopes on the major allergic proteins such as *Der p I* and *Der p II*. The general approach then has been to identify mAbs singly or in combination which are effective for suppressing DTH responses to individual *Der p* (and *Der f*) proteins. Further selection of NUB combinations may also be employed for suppression of DTH responses to mixed dust mite allergens such as those contained in crude extracts (DMA).

A DTH response is elicited in BALB/c mice sensitized with DMA (10 μ g) and challenged in the ear with DMA 5 days later. There is a significant ($p < 0.01$) increase in ear swelling in DMA-sensitized mice compared to nonsensitized controls DTH responses are be similarly detected in BALB/c mice sensitized with DMA followed by ear challenge with *Der p I* protein preparations.

8.2 Suppression of *Der p I* induced DTH Response in Mice Treated with Anti-*Der p* mAb

Mice sensitized with DMA extract (10 μ g) and challenged in the ear 28 days later elicit a delayed type hypersensitivity response as assayed by ear swelling. This response is significantly reduced when mice are treated after sensitization with DMA with 10 to 50 μ g

anti-Der p I mAb H11 (Figure 12 and Table 7).

Prophylactic experiments show that treatment with mAb H11 following sensitization with DMA suppresses the development of DTH responses following ear challenge with Der p I (10 µg) as shown in Table 7. When DMA sensitized mice are treated with H11 (3 x 20 µg days 4, 7 and 14) and ear challenged, on day 28 significantly reduced ear swelling is observed. A significant reduction in DTH responses to mAb H11 treatment is obtained using a range of mAb doses from 1 µg to 1 mg given up to 5 times. This response is further enhanced when mAb H11 is administered with aluminum hydroxide as adjuvant ("ALHYDOGEL 85"; SuperPhos Biosector a/s Vedbaek, Denmark).

15 **8.3 Decrease in T cell reactivity by *in vitro* parameters**

In addition, lymphocytes from these sensitized animals are isolated from lymph nodes or spleen, and single cell suspensions are placed in culture and stimulated with either whole DMA or Der p I and Der p II. T cell activation is measured either by incorporation of radiolabeled thymidine, or by production of IL-2. Those animals injected with Ab1 have significant decrease in either of these activation markers as compared to sensitized animals without this treatment.

30

35

TABLE 7
Suppression of Delayed Type Hypersensitivity
Responses to *Der p I* in Mice by treating with
Anti-*Der p I* Antibodies

5 Treatment regimen:

	Reagent	Dose μ g	Schedule Days	Increase in ear swelling (mm $\times 10^{-2}$) following challenge with <i>Der p I</i> (10 μ g) ²
10				
				120 \pm 11
15	mAb H11 ³	20	4,7,14	250 \pm 9
	mAb 198 ⁴	20	4,7,14	98 \pm 14
	mAb H11	10	4,7,14	48 \pm 7
	mAb H11 in Alum	10	4,7,14	13 \pm 5
20	mAb H11 in Alum	5	4,7,14	22 \pm 8
	mAb 198 in Alum	10	4,7,14	92 \pm 12

¹ With respect to DMA sensitization (Day 0)

² Challenge on Day 21

25 ³ Anti-*Der p I* mAb

8.4 Assessment of down regulation of IgE anti-DMA response

8.4.1 SCID mouse model

30 Down-regulation of antigen processing by injection of anti-DMA monoclonal antibodies can be assessed by use of mice genetically bred to lack an immune system severe combined immune deficiency (SCID) mice. The animals do not have the capacity to be sensitized, but can mount an anamnestic response. These animals are repopulated with human immune systems using either peripheral blood

35

mononuclear cells, lymphocytes from tonsils, or a mixture of T cell clones taken from disease specific organs such as the lung in asthmatics, and mixed with peripheral blood mononuclear cells. Optimally these lymphocytes
5 should be taken from atopic patients suffering from allergic reactions to the target antigen, such as dust mite, as manifest by antigen-specific IgE, skin test positivity to the antigen, and clinical symptoms indicating ongoing exposure. Alternatively subjects may
10 be deliberately exposed to the allergen prior to donation of the tissues.

The SCID mice are injected intravenously with 10×10^6 lymphocytes and blood is drawn after 1 week and measured for total and antigen specific IgM, IgG, and
15 IgE, to verify engrafting. They are then injected with either DMA specific monoclonal antibody of an irrelevant monoclonal antibody of the same subclass, and challenged with antigen, 10-100 μ g without adjuvant 1-3 days later. The antigen challenge is repeated again 2 weeks later,
20 and blood is drawn weekly for up to 6 weeks thereafter.

8.4.2 Normal mouse model

In a second model, BALB/c or A/j mice are exposed to DMA either as the purified antigen, or as the
25 semipurified extract. The exposure can either be as an injection with and without adjuvant, in a dose range of 20 μ g to 1 mg given as 1-3 injections 1 week apart, or as the aerosolized antigen, given in a nebulized form up to 1 mg/day for 10 days. Following exposure the IgG and IgE
30 anti-DMA antibody response is determined.

In order to determine if a candidate mAb is able to down-regulate the response, the mAb is either given prior to sensitization, at a dose from 0.01 μ g to 100 μ g, with or without adjuvant, as multiple injections ranging from
35 1-10, up to 1 week prior to sensitization; or after sensitization with or without adjuvant at periods ranging

from immediately after the last antigen exposure up to 1 month after exposure. In some cases the latter group of animals may be rechallenged to investigate the effect of such treatment on the secondary response. Results are shown in Table 8.

TABLE 8

Suppression of *Der p I*-specific IgE Responses in Mice by Treatment with Anti-*Der p I*¹

10

Expt	Schedule of mAb H11 Treatment (days) ³	mAb H11 μ g	Serum IgE Titer ²
1	—	—	60 \pm 10
15	4, 7, 14	3 x 10	23 \pm 7
2	—	—	75 \pm 12
	7, 14, 21	3 x 5	43 \pm 11
20	7, 14, 21	3 x 10	18 \pm 12
3	—	—	68 \pm 12
	14, 21, 28	3 x 10	21 \pm 4

25

In these experiments, mice were immunized 3 to 5 times at monthly intervals with *Der p I*. Treatment with mAb H11 was conducted 7 days after final treatment with *Der p I* extract. Serum assays for IgE titer were performed 14 days after final treatment with mAb H11.

30

¹ Mice Immunized 3 to 5 times at monthly intervals with *Der p I*

² Serum assay 14 days after final treatment with MAB H11

³ MAB H11 treatment 7 days after final treatment with *Der p I* extract

8.4.3 Aerosol-sensitized mouse model

In a third model to measure the ability of the mAb to down-regulate the response either BALB/c or A/j mice or Brown Norway rats are treated with nebulized allergen for 30 minutes each week for 6 weeks, with molecules in the range of less than 1 μ M, and tested for production of total and antigen specific IgG and IgE (McMenamin, C., et al. Immunology, 77:592-596, 1992). To test the ability of the candidate mAb to significantly down-regulate either of these antigen-specific classes, the mAb can be given as in the example above, either before or after antigen challenge, with or without adjuvant.

In both cases the IgE and IgG response is determined by the general methods set forth in Section 9 below, except that the antigens will be *Der I* or *Der II* instead of *Lol p I*. In addition, animals are injected intradermally with 1-100 μ g of antigen and the cutaneous response is determined 30 minutes later as a measure of IgE response.

20

9. Example: Inhibition of Allergic Response to Dust Mite Allergens

9.1 Development of an IgE Dust Mite Murine Model to Detect Serum IgE Responses

A model system exists on which to test the IgE-mediated allergic reaction to dust mite. In this model, ovalbumin (OVA) is aerosolized, and used to sensitize BALB/c mice by inhalation. These animals develop immediate hypersensitivity skin test reactions upon challenge, and sera are able to transfer passive cutaneous anaphylaxis. The model is antigen specific. IgE sensitization can be transferred with peribronchial lymph nodes of sensitized animals (Saloga et al. J. Clin. Invest., 91:133-140, 1993). Bronchospasm in the animals was demonstrated following electrical field stimulation (Larsen et al., J. Clin. Invest., 89:747-752,

35

1992), although there was no acute inflammatory cells including eosinophils (Renz et al. J. Allergy Clin. Immunol., 89:1127-1138, 1992).

5 The OVA system represents a good model for assessing immunotherapeutic agents for treatment of dust mite allergy. Adjuvant is not used in stimulating allergic responses, thus avoiding nonspecific T cell and other inflammatory responses, as well as alteration of cellular functions produced by these agents. The aerosolized route of sensitization is similar to the human situation, and allows specific measurement as to the effect on the airways.

15 This aerosolized allergen model with *Der p I* or *Der p II* has been used to demonstrate that immunization of BALB/c mice with vaccines containing murine anti-*Der p I* or *Der p II* monoclonal antibodies suppresses anti-*Der p I* or *Der p II* IgE antibody responses. By way of illustration experiments using anti *Der p I* mAb 2C7 to suppress *Der p I* serum IgE responses are described.

20

9.1.1 Sensitization with Aerosolized Dust Mite Allergen

25 Particles of 2-3 microns, small enough to reach the lower respiratory tract (Yu, Powder Tech., 21:55, 1978) are generated by a jet nebulizer ("MICRO CIRRUS", Intersurgical, Middlesex, UK) under a continuous pressure of 29 PSI. Female BALB/c mice (8-12 weeks old) are sensitized by ultrasonic nebulization, of 1% whole dust mite extract in sterile phosphate buffered saline pH 7.3, based on the procedures developed by Gelfand and associates (Renz et al., J. Allergy Clin. Immunol., 89:1127-1138, 1992). Up to 5 mice are placed in a sensitization chamber, and the dust mite solution is aerosolized into the inlet port. They are exposed to the aerosolized allergen 20 minutes for 10 days. Controls include PBS as a negative control, and Ovalbumin (OVA 1%)

35

and crude, soluble rye grass extract (*Lol p*) obtained from a commercial source as positive controls.

9.2 Immune Response Against *Der p I*

5 The *Der p I* specific IgE response is measured by passive cutaneous anaphylaxis. Positive results are confirmed by measurement of immediate cutaneous hypersensitivity, and measurement of total and antigen specific IgE by ELISA. Airway responsiveness is measured
10 by testing for increased bronchial resistance to allergen challenge.

9.2.1 Passive Cutaneous Anaphylaxis (PCA)

Inbred Sprague-Dawley rats (6/group) are injected
15 intradermally with 100 μ l of a range of dilutions of serum samples. In this assay up to 8 intradermal sites are available so that a series of dilutions of test sera can be injected. In controls, rats are injected with serum from control mice exposed to aerosolized saline or
20 other allergens, e.g., ovalbumin or rye grass *Lol p I*. Forty eight hours later rats are injected intravenously with 10 to 50 μ g of purified *Der p I* (dose determined in pilot studies) in admixture with 1 ml Evans blue dye (1%). Rats are killed 30 minutes later and the response
25 is determined from the infiltration of Evans blue as visualized on the inverted skin surface. A positive skin response is defined as a wheal of greater than 0.3 cm in diameter. A test serum titer is determined by comparison with controls.

30

9.3 Suppression of Anti-*Der p I* IgE Responses

BALB/c mice (10/group) were immunized with mAb 2C7 in alum using conditions defined to stimulate anti-idiotypic antibodies. Control mice (10/group) received
35 an irrelevant mAb (anti-CEA mAb 337) or saline. Following immunization mice were exposed to aerosolized

dust mite extract or saline as already described and blood samples were taken two days after completing the treatment protocol. Serum samples from groups of 10 mice were pooled and tested for IgE specific antibody by the passive cutaneous anaphylaxis assay and by ELISA assay.

9.3.1 Passive Cutaneous Anaphylaxis Assay

In the experiment summarized in Table 9, treatment of mice with mAb 2C7 prior to sensitization with aerosolized *Der p* produced a 6-fold reduction in the IgE response to *Der p I* as measured by passive cutaneous anaphylaxis. In comparison, mice treated with control mAb 337 still generated anti-*Der p I* IgE antibodies (Titer 1/128) when exposed to aerosolized *Der p*.

9.3.2 Determination of Murine Serum Anti-*Der p I* IgE Antibody Levels

Anti-*Der p I* serum IgE antibody titers are measured by ELISA in the following procedure:

1. ELISA microtiter plates are coated with goat anti-human IgE antibody.
2. The microtiter plates are washed and blocked with casein buffer.
3. Human serum from atopic dermatitis subjects is added and incubated overnight at 4° C.
4. The microtiter plates are washed with PBS--Tween.
5. BALB/c mouse antisera raised against mAb--Keyhole limpet hemocyanin is added to microtiter plates. For a control, normal BALB/c mouse serum is used.
6. The microtiter plates are washed.
7. The bound mouse antibody is detected by reaction with goat anti-mouse IgG labeled with horseradish peroxidase.
8. The substrate ABTS is added.

9. Color development is determined at OD 405 nm
Standard ELISA reagents are described in Section 6.

TABLE 9

5

Inhibition of Anti-Der *p I* IgE Antibody
Responses in Mice Immunized with Anti-Der *p I*
mAb 2C7 Passive Cutaneous Anaphylaxis Test (PCA)

10	Group	Immunizing mAb*	Sensitization by Aerosolized Exposure to:	PCA Titer to Der <i>p I</i>
	1	2C7	Der <i>p</i>	1:4
	2	2C7	PBS	0
	3	Control mAb 337	Der <i>p</i>	1:128
15	4	Control mAb 337	PBS	1:2
	5	Saline	Der <i>p</i>	1:256

*5 injections of mAb (10 μ g in alum) delivered
subcutaneously at two week intervals; mAb 337 is
20 directed against carcinoembryonic antigen.

Treatment of BALB/c mice with mAb 2C7 in alum did
not influence the induction of anti-ovalbumin IgE
antibody responses in mice receiving aerosolized
25 ovalbumin. In these experiments the PCA titer in mice
treated with either saline or mAb 2C7, or mAb 337, and
then exposed to aerosolized ovalbumin were 1/512 and
1/256 respectively.

30 9.3.3 ELISA Assay Anti-Der *p I* IgE Antibody

Serum samples from mice exposed to aerosolized Der *p*
were assayed for Der *p I*-specific IgE antibodies using an
ELISA assay (described *supra*). Briefly, serum samples
undiluted and diluted 1/2 to 1/50 were added to ELISA
35 microtiter plates coated with Der *p I*. After incubation
and washing, bound mouse serum IgE was detected with rat

anti-mouse IgE horseradish peroxidase conjugate. A serum dilution titer was determined from the ELISA OD readings (405 nm) in comparison with background OD values with microtiter plates exposed to normal mouse serum.

5 In the example summarized in Table 10, mice treated with saline or control anti-CEA mAb 337 developed anti-*Der p I* IgE antibodies when exposed to aerosolized *Der p* preparation (serum titers 1/50 and 1/40 respectively). This response was reduced some ten-fold in mice immunized
10 with anti-*Der p I* mAb 2C7 in an alum formulation (serum titer 1/4). In control studies immunization with mAb 2C7 in alum did not influence the stimulation of anti-ovalbumin IgE antibodies following exposure of mice to aerosolized ovalbumin. In one example the serum anti-IgE
15 titer as determined by ELISA in mice immunized with mAb 2C7 or mAb 337 (1/400 in each case) was comparable to that obtained in mice initially treated with PBS (titer 1/500).

20 9.3.4 Results

 In the example described above, treatment of BALB/c mice with anti-*Der p I* mAb 2C7 in an alum formulation led to the suppression of the ability of mice to generate anti-*Der p I* IgE antibodies on exposure to aerosolized
25 dust mite extracts (*Der p*). In further examples anti-*Der p I* mAb H11 suppressed anti-*Der p* serum IgE responses and mAb 2H5 suppressed anti-*Der p II* IgE responses.

30

35

TABLE 10

Inhibition of Anti-Der p I IgE Antibody
Responses in Mice Treated with Anti-Der p I
mAb 2C7: ELISA Assay

5	Group	Immunizing mAb*	Sensitization by Aerosolized Exposure to:	Serum Anti- Der p I IgE Titer
	1	2C7	Der p	1/4
	2	2C7	PBS	0
	3	Control mAb	Der p	1/40
10		337**		
	4	Control mAb	PBS	0
		337**		
	5	PBS	Der p	1/50

(*) 5 injections of mAb (10 μ g in alum) delivered
subcutaneously at two week intervals;

(**) mAb 337 is directed against carcinoembryonic
antigen.

10. Example: Use of anti-urushiol monoclonal antibodies
(Ab1) to down regulate the T cell response
to poison oak and ivy allergy

Poison oak/ivy allergy is a delayed type
hypersensitivity (DTH) response to allergen (urushiol) in
the oil of the plants. The natural allergen is a mixture
of 3-n-alkylcatechols with a C15 or C17 side chain either
fully saturated e.g. 3-n-pentadecylcatechol, PDC) or
having 1 to 3 unsaturated bonds (Figure 25) (Symes and
Dawson, 1954, J. Ann. Chem. Soc., 76:2959-2963). In the
initiation of an allergic response the allergen first
undergoes quinone formation. The quinone then undergoes
reaction with cell proteins through sulfhydryl or amino
groups and these products initiate the allergic response.

10.1 Preparation of 3-n-Alkylcatechol Conjugates for Use as Immunogens

Urushiol and related 3-n-alkylcatechols produce T cell responses; antibodies have never before been demonstrated. Thus, to generate anti-urushiol antibodies, it has been necessary to synthesize analogs capable of stimulating B cell responses. These have been synthesized by several routes, a preferred pathway being reaction of quinones of urushiol and PDC with proteins, peptides, and amino acids.

10.1.1 Reaction of auto-oxidized urushiol to proteins

The conjugation procedure is based upon that described by Liberato et al., 1981, J. Med. Chem., 24:28-33. Urushiol, 1.56 μ M dissolved in 1 ml acetone is placed into a conical flask and dried onto the inner surface under a stream of nitrogen. Protein solution (serum albumen 100 mg in 10 ml phosphate buffered saline pH 7, or keyhole limpet hemocyanin in sodium hydrogen carbonate buffer pH 8.4) is added. Alternatively, urushiol may be added drop-wise e.g. 0.2 ml urushiol added to KLH solution 20 mg in 2 ml of buffer pH 8.4. The heterogeneous mixture is stirred open to air at room temperature for up to 48 hours. During this time the urushiol undergoes o-quinone formation and protein coupling to yield a red-brown colored solution. The solution is chromatographed (e.g. on Sephadex G25) to separate the protein conjugate. In a typical example, the absorbance (480 nm) of the albumin solution increased from zero to 0.59. The final solution is sterilized and stored at 4° C.

10.1.2 Conjugation of Alkylcatechol to Protein through its n-Acetyl Cysteine Intermediate to Common Protein Carriers

Quinone derivatives of urushiol and 2-n-pentadecylcatechol (PDC) are prepared by oxidation of urushiol or PDC with silver oxide using procedures described by Liberato et al. *ibid.* to yield n-acetyl-S (2,3)-dihydroxy-4-pentadecylphenyl cysteine (PDC-n-acetyl cysteine). The purity of the product was assessed by thin layer chromatography, elemental analysis and NMR spectroscopy to conform with published data (*ibid.*). The quinone derivative of urushiol is similarly reacted with N-acetyl cysteine.

10.1.3 Conjugation of 3-n-pentadecylcatechol (PDC) to Polyaspartic Acid

Conjugation of PDC to polyaspartic acid is effected through a carbamide complex. PDC (10 mg) was dissolved in dioxane (1 ml). Dicyclohexylcarbodiimide (DCC: 2 mg) was added, and reacted for 18 hours at 4° C. The complex was then added to acid (5 mg; 41.6 μ M) in phosphate buffered saline pH 5 and after 30 minutes the conjugate isolated by Sephadex G25 chromatography. Spectroscopy analysis (277 nm) indicated the mean substitution ratios was 42.8 μ mol/mg.

10.2 Production of Anti-Urushiol Monoclonal Antibodies and TCRs

Methods for producing murine monoclonal antibodies have been described (Section 5). BALB/c mice were immunized on day 0 with PDC-n-acetyl cysteine (50 μ g) subcutaneously in Freund's complete adjuvant followed by an intravenous boost dose of PDC-n-acetyl cysteine on day 7. Spleen cells were harvested on day 11 and fused with myeloma P3 NSO cells. Hybridoma clones are selected as described *supra*.

Anti-urushiol producing monoclonal antibodies are identified by ELISA assays. Briefly, polyvinyl microtiter plates (Falcon) are coated with urushiol or PDC-conjugates such as PDC and urushiol-n-acetyl cysteine and PDC-polyaspartate. Hybridoma supernatants are added, incubated 45 minutes, washed and bound with mouse immunoglobulin deleted with horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin. This methodology yielded mAb 991/81/7, of the IgM isotype from a mouse immunized with PDC-n-acetyl cysteine. mAb 991 is purified from hybridoma culture supernatants by ammonium sulfate precipitation and fractionation by Sephacryl S300 gel filtration chromatography.

10.3 Immunoreactivity of mAb 991

The immunoreactivity of mAb 991 is initially determined by its binding in ELISA assay to urushiol and PDC conjugates but not to carrier molecules. The specificity of mAb 991 is further defined by the inhibition of its binding to urea-n-acetyl cysteine by compounds related to 3-n-alkylcatechols. This is illustrated by Figure 26 which summarizes inhibition of mAb 991 to urea-n-acetyl cysteine. Thus compounds such as catechol and catechin hydrate with features of the catechol moiety inhibits mAb 991 binding. From these experiments mAb 991 is described as reacting with the catechol structure.

10.4 Down-regulation of Delayed Type Hypersensitivity Responses to Urushiol

The use of anti-urushiol monoclonal antibodies to suppress allergic responses to urushiol has been evaluated using a murine model (Dunn et al., 1982, Cell Immunol., 68:377-388). BALB/c mice are sensitized by application of urushiol or PDC (2 - 4 mg) to the abdomen in acetone (100 μ l). Sensitization to the allergen is

then detected from day 4 up to day 42 by application of urushiol or PDC (50 μ g in 10 μ l acetone) to the dorsal side of one ear. The other ear is treated with acetone only (10 μ l). Ear thickness is then detected using a
5 sensitive pressure micrometer (Mitutoya, Japan) and the difference between allergen-challenged and control ears determined.

In a prophylactic approach (Figure 27) mice are treated by a single or multiple intravenous injections of
10 mAb using a range of doses (1 to 25 μ g). Mice are then sensitized to the allergen and challenged on the ear. As an example of this approach, mAb 991 treatment (3 times, 10 μ g) suppressed to DTH response to urushiol compared to control mice receiving an unrelated IgM mAb (B55: anti-
15 breast cancer).

In therapeutic tests mAb is given after sensitization to urushiol or PDC. This is illustrated in Figure 28 where urushiol sensitized mice received mAb 991 as a single dose (10 μ g) intravenously 21 days after
20 sensitization. When ear challenged on day 42 (50 μ g urushiol), the DTH response was significantly reduced.

mAb in adjuvant are even more effective than mAb alone. This is illustrated by experiments where urushiol sensitized mice were treated on day 7 and 14 with mAb 991
25 (1 μ g) in complete and then incomplete Freund's adjuvant. When ear challenged on day 21 the DTH response was reduced by almost 80 per cent compared to that in control mice receiving control mAb (Figure 29).

30 10.5 Anti-urushiol antibodies

Immunoglobulin molecules (Ig) in the form of B lymphocytes to urushiol are induced in mice, rats, rabbits, guinea pigs, or the like by techniques well known to those skilled in the art, wherein such
35 techniques employ urushiol protein carrier conjugates as previously described. For example, animals are immunized

with one or more injections of urushiol-protein conjugates. These are administered by any of several routes including intravenous, subcutaneous, intramuscular and intradermal injection with or without an adjuvant capable of potentiating host immune response. An example of such an adjuvant is complete or incomplete Freund's adjuvant. In some instances, immunoglobulin molecule responses to urushiol are enhanced by deleting, immune responses to carrier protein. This is accomplished by elimination of responses to carrier protein by treatment with lymphotoxic drugs using the general procedures described by Thomas, W.A., J. Immunol. Methods, 1987, 97:237-243. Briefly, animals are immunized with-carrier protein. At intervals after immunization, animals are treated with cytotoxic drugs, e.g., cyclophosphamide (40 mg/kg body weight) to eliminate lymphocyte clones producing immunoglobulin-molecules to carrier protein. Animals are then allowed to recover from this treatment and then immunized with urushiol-protein conjugate. Immunoglobulin molecules are derived from serum of immunized animals. In some cases, mouse and/or rat monoclonal antibodies are produced by hybridoma production with antibody producing cells e.g., spleen cells from immune donors.

25

10.5.1 Production by simple immunization with urushiol-carrier with or without adjuvant

Adult BALB/c mice are immunized with urushiol-key hole limpet hemocyanin conjugate (uru-KLH). In a typical protocol, the first dose (day 0) of 100-400 μ g protein conjugate in complete Freund's adjuvant (CFA) emulsion is injected intraperitoneally. The second immunization (day 10-14) is injected intraperitoneally. This consists of uru-KLH (100-400 μ g) emulsified in incomplete Freund's adjuvant (ICFA). A third dose of uru-KLH is given without adjuvant by intraperitoneal injection 10-14 days

later. Further doses are given at 2-3 week intervals until immunoglobulin molecules to urushiol are detected in serum.

5 10.5.2 Production of anti-urushiol responses
 using additional immune manipulation

Adult BALB/c mice are immunized intraperitoneally with human serum albumin (10 mg) in complete Freund's adjuvant. Three days later mice are treated with
10 cyclophosphamide (40 mg/kg intraperitoneally). This treatment destroys lymphocytes producing Ig to human serum albumin (HSA). After resting for 2-25 days, mice are then immunized with uru-HSA (100-200 μ g) in complete Freund's adjuvant. As in Example A, *supra*, the
15 immunization is given at intervals with and without Freund's adjuvant (complete or incomplete) until immunoglobulin molecules are detected in serum.

 10.5.3 Use of urushiol alone as B cell
 immunogen

20 Adult BALB/c mice are immunized by multiple paintings of free urushiol in acetone, up to about 2.5 mg, on the abdomen and sera is repeatedly drawn for testing. Paintings are continued until anti-urushiol
25 antibodies are found in the serum.

 10.5.4 Use of lymphocytes from sensitized
 humans

Sensitized humans are challenged with urushiol by epicutaneous administration of 0.1-5 μ g of urushiol in
30 acetone. Sera are tested for anti-urushiol antibodies, and lymphocytes collected from cutaneous lesions or from peripheral blood are used to produce hybridomas by techniques well known to those skilled in the art.

35

10.6 Production of Anti-Urushiol Monoclonal Antibody (Ab1)

10.6.1 Murine monoclonal antibodies

Methods of making murine monoclonal antibodies to an antigen of interest are well known to those skilled in the art and have been described previously. (Goding, *supra*) For example, spleen cells from urushiol-immune mice are obtained by disaggregation of spleen tissue in serum-free medium (RPMI 1640). Mouse myeloma cells (P3-NS1, Sp 2/0, X63-Ag8.653, NSO/1, or the like) are harvested when sub-confluent and still in exponential growth and washed in serum free RPMI 1640 medium. Cell preparations are mixed in a ratio of 1 myeloma cell to 5 spleen cells and pelleted by gentle centrifugation in conical-bottomed flasks. The supernatant is removed, cell pellet loosened and polyethylene glycol (PEG 1500) added, 1.8 ml over 2 minutes. The cells are rapidly hand rotated and allowed to stand 2 minutes. Serum-free RPMI 1640 medium is added drop-wise (1 ml over 1 minute then 20 ml over 5 minutes) while rotating the tube, then centrifuge. The supernatant is removed and the cell pellet suspended in RPMI 1640 with 15% fetal calf serum and hypoxanthine, methotrexate, and thymidine.

Further growth medium is added and the mixture is plated at 5×10^3 myeloma cells, 100 μ l/well in 96 well flatbottomed microtiter plates on a feeder layer of rat peritoneal exudate cells. The cells are incubated at 37°C in 5% CO₂ (Galfre, et al., Nature, 1977, 226:550-552). Fusion plates are examined microscopically every 3-4 days and fed by aspirating half of the supernatant from wells and replacing with fresh growth medium. Wells having hybridoma colonies visible to the naked eye are tested for Ig. Murine monoclonal antibodies are obtained from tissue culture supernatants of hybridoma cells grown *in vitro*. These are purified in various ways, the preferred method being affinity chromatography on a

Sepharose-protein A column. In this procedure hybridoma cell supernatants are passed through chromatography columns containing Sepharose-protein A. The bound Ig is then eluted from the column using appropriate media.

5

10.6.2 Production of human monoclonal antibodies

10.6.2.1 From murine monoclonal antibodies

10 The use of murine monoclonal antibodies to produce human monoclonal antibodies by gene fusion techniques are well known to those skilled in the art. They are of two types. The first utilizes the variable region of the murine antibody gene cloned onto a human antibody backbone. The second uses the idiotypic region of the murine monoclonal antibody gene cloned onto a human backbone. (See e.g., Morrison et al., EPO EP173494 (3/5/86); Neuberger et al., PCT WO-8601533 (3/15/86); Robinson et al., PCT WO-8702671; Morrison et al., Proc. Natl. Acad. Sci., USA, 1984, 81:6851-6855; Boulianne et al., Nature, 1984, 312:643-646; Neuberger et al., Nature, 1985, 314:268-270).

20 Chimeric antibody production involves isolation of messenger RNA (mRNA) from a murine B cell hybridoma producing the relevant monoclonal antibodies. This is then cloned and a complementary DNA library is prepared. See, e.g., Perbal, B., A Practical Guide to Molecular Cloning, 2d ed., John Wiley & Sons (1988), which is incorporated herein by reference. The required variable regions of the light and heavy chain genes are then identified with appropriate probes. These are sequenced and made compatible with a constant region gene segment which is also obtained by construction of cDNA preparations and cloning. Heavy and light chain coding sequences are constructed by linkage of the cloned specific immunoglobulin variable (V) gene segment to

35

cloned human constant gene sequence. On expression in prokaryotic and eukaryotic cells, chimeric antibodies are produced containing the antibody specificity of the mouse monoclonal antibody but with most of the molecule
5 comprising human Ig amino acid sequences.

10.6.2.2 By fusion of human B
lymphocytes with a mouse-
human heterohybridoma

Human lymphocytes producing a desired antibody are
10 fused with a mouse-human heterohybridoma cells to produce humanized monoclonal antibodies. The human lymphocytes may be taken from an active urushiol skin lesion or from the peripheral blood.

In one embodiment, mouse/human heteromyeloma cells
15 such as EL41 (deposited on May 16, 1990 with the European Collection of Animal Cell Cultures and having Accession No. 90051602) as described by Austin et al., Immunology, 1989, 67:525-530, are fused with human lymphocytes reactive with urushiol using polyethylene glycol
20 according to the method of Galfre et al., *supra*. For example, lymphocytes are placed in 30 ml plastic universal bottles and washed twice with growth medium such as Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories) and the number of lymphocytes is
25 determined by hemocytometer counting. EL41 cells are harvested, washed, and counted in a similar fashion. The two cell populations are then mixed at a lymphocyte:fusion partner ratio of 2:1 and washed for a third time. Following centrifugation the medium is
30 discarded and 0.8 ml of 50% PEG pipetted over a 1 minute period onto the cell pellet with gentle agitation. The mixture is left for 1 minute then 1.0 ml of medium is added over a 1 minute period followed by the addition of 20 ml of medium added slowly over a 5 minute period. The
35 cells are then centrifuged at 1200 rpm for 5 minutes, the medium discarded, and the cells resuspended by gentle

pipetting in a 2 ml pipette. Twenty ml of selection medium (DMEM + 10% FCS + 10^{-4} M hypoxanthine + 10^{-5} M methotrexate + 10^{-6} M thymidine) is finally added to the cell suspension and the cells aliquoted into two 96 well
5 tissue culture microtiter plates containing rat peritoneal exudate cells (2.5×10^3 /well) as a feeder layer. An additional 100 μ l of media is also added to each well. The selection medium is changed every 72 hours until resulting hybridomas have reached 50%
10 confluence then every 48 hours. The growth of hybridomas is regularly checked and when the colonies cover 50% of the well the supernatant is analyzed for Ig. Hybridomas producing Ig of interest are immediately cloned. Established hybridomas are frozen down in aliquots of 20
15 $\times 10^6$ cells/ml in 95% FCS and 5% dimethylsulfoxide (DMSO) and stored in liquid nitrogen.

10.7 Production of Anti-Urushiol T Cell Receptors For Use As Immunogens

20 Mice are vaccinated against haptens (oxazolone, DNFB), including urushiol, by immunization with T cells derived from lymph nodes of hapten sensitized donors. Mice are vaccinated by immunization with urushiol specific T cells. These may be cloned 20 T cells, T cell
25 hybridomas, or peptides mimicking the TCR of the specific T cells.

10.7.1 Production of urushiol specific T cell lines from sensitized mice

30 BALB/c mice are sensitized following application of urushiol (0.01-5 mg) in acetone onto the dorsal skin. T cells from sensitized mice are obtained from several sources including lymph nodes or urushiol-induced cutaneous lesions and expanded in culture by *in vitro* stimulation with urushiol or urushiol-polymer conjugates
35 using stimulation procedures well known to those skilled in the art. Urushiol specific T cell clones or T cell

hybridomas are obtained by fusion of T cells with murine thymoma cells (BW 5147 cells as described by Bigby et al., J. Immunol., 1989, 143:3867-3872). These cells produce immune responses to urushiol specific TCR. Such cells have utility as T cell vaccines which may be one of several types including viable or attenuated T cells and T cells modified by cross linking with agents such as glutaraldehyde (Cohen, I.R. and Weiner, H.L., Immunol. Today, 1988, 9:332-335). The TCR of the urushiol responding-T cell clone is identified by reaction with monoclonal antibodies which detect variable segments of the TCR (Kappler et al., Nature, 1988, 332:35-40).

In another approach, messenger RNA or DNA from cloned T cells reacting with urushiol are probed either directly or after amplification by polymerase chain reaction by specific hybridization with nucleic acid probes for TCR gene families using hybridization methods well known to those skilled in the art. The TCR sequence (in part) is obtained from the amplified DNA or RNA. Regions of TCR amino acid sequences are selected on the basis of their immunogenic or antigenic properties identified using algorithms to predict T cell helper/cytotoxic determinants (Rothbard, J.B. and Taylor, W.R., EMBO J., 1988, 7:93-100). For example, peptides containing 14 or 15 amino acids are predicted to contain specific TCR sites for urushiol. Peptides of selected amino acid composition are then synthesized using standard procedures well known to those skilled in the art. Synthetic peptides are then used to vaccinate animals.

10.7.2 TCR produced from immune human donors

T lymphocyte clones are established from peripheral blood mononuclear cells (PBMC) or contact dermatitis lesions derived from urushiol sensitized human subjects

by methods generally described by Kalish and others.
(Kalish, R.S. and Morimoto, C., J. Clin. Invest., 1988,
82:825-832; J. Invest. Dermatol., 1989, 92:46-52). PBMC
5 isolated from venous blood by "FICOLL-HYPAQUE" separation
are cultured in IL-2 containing media and sensitized or
expanded by inducing proliferation *in vitro* by the
addition of urushiol in dimethylsulfoxide or on
10 autologous PBMC (Byers et al., J. Clin. Invest., 1979,
64:1437-1448). Other growth factors such as IL-4 may be
used (Paliard et al., J. Immunol., 1989, 43:452-457).
Following culture for a period of time to establish
15 satisfactory cell growth, cells are cloned by limiting
dilution using standard cell culture techniques. Cells
are then grown and positive wells expanded. This phase
20 may include further stimulation of cells with urushiol
and autologous irradiated (5000R) PBMC as feeder cells.
T cell clones are then established by standard
techniques. Alternatively, T cell hybridomas may be
25 employed as a source of TCRs. The T cell vaccine may be
composed of one or more formulations including viable or
attenuated T cells and T cells modified by cross linking
agents, e.g. glutaraldehyde (Cohen, *supra*). Ideally, T
30 cell vaccines should be of autologous origin since
responses are generally restricted by the MHC component
of the TCR (Schwartz, R., Ann. Rev. Immunol., 1985,
3:237-261). Since broad MHC restriction is, however,
35 involved in the recognition of certain haptens and

antigens (Panina-Bordignon et al., Eur. J. Immunol., 1989, 19:2237-2242). An alternative approach is to administer vaccines composed of pooled preparations of
5 cloned T cells derived from a number of allogeneic donors.

In another approach, specific antigen binding regions of the TCR may be identified, and the peptide
10 comprising that region synthesized as described previously for use as an immunogen.

11. **Suppression of the IgG antibody response to an exogenous antigen, Ricin A Chain (RTA) by monoclonal anti-RTA antibodies**
15

11.1 Production of Ab1 to RTA

BALB/c mice received a single intravenous injection of an immunotoxin composed of the murine monoclonal
20 antibody 791T/36, conjugated by a disulfide bond to RTA. As shown in Figure 1, significant titers of anti-RTA antibody were noted 7 days after injection, and continued to increase by day 30.

Monoclonal antibodies were generated against RTA
25 using splenocytes from BALB/c mice immunized with the immunotoxin 791T/36, fused with the myeloma cell line P3NS1, and cloned and characterized as previously described. Five mAbs were selected for further study. Three (596/134, 596/192, and 608/7) reacted equally well
30 with native RTA, but not with RTA modified by heating at 56° C, indicating they are directed against peptide epitopes on the molecule but not to oligosaccharide structures on the polypeptide Figure 2. RTA is known to carry one or two mannose containing residues. Of these,
35 monoclonal antibodies, mAb 596/134 and 608/7 had the highest affinity as indicated by their continued high

binding at concentrations below 1 $\mu\text{g/ml}$. Two other mAbs, 608/9 and 608/42 showed weaker binding to the native molecule, but very good binding to deglycosylated RTA, suggesting they recognize a cryptotope which is covered by the sugar residues in the native molecule (Figure 3).

11.2 Down-regulation of IgG with Ab1

BALB/c mice were immunized with immunotoxin 791T/36-RTA, and then 24 hours later were treated either with a single injection of 100 μg given intravenously with a anti-RTA mAbs, or with a control mAb, mAb 365, directed against carcinoembryonic antigen, which does not cross react with RTA. Animals were bled 14 days after initial antigen injection and the IgG anti-RTA serum titers were measured by ELISA, and suppression was compared with that of the control animals treated with the anti-CEA antibody. Three of the five mAbs, 608/7, 596/134 and 596/192 significantly suppressed the anti-RTA response (Figure 4). The effect was most pronounced with mAb 608/7 and 596/134, producing 71 and 73% inhibition respectively. Titration of the response with mAb 608/7 (25-50 μg) demonstrated that a single dose of antibody (Figure 5) resulted in 40-60% reduction in anti-RTA antibody titer.

Treatment with anti-RTA mAb 608/7 between 2 and 24 hours following immunotoxin treatment produced significant inhibition (p less than 0.001) of anti-RTA antibody production (Figure 6). In other experiments (Figure 7), mAb 608/7 was effective up to three days after immunization. In other experiments, (Figure 8), mAb 608/7 was also effective when administered up to 7 days before immunotoxin treatment, and produced very effective inhibition of anti-RTA responses.

Immunotoxins are rapidly removed from the circulation, with a $T_{1/2}$ of about 20 minutes. This short half life is due to the mannose residues on the

RTA, which bind to receptors on the Kupffer cells of the liver. Since antibody is still capable of down-regulating the IgG anti-RTA response when given as late as 3 days after antigen exposure, it cannot simply be preventing the antigen from reaching the antigen processing cells, since none remains in the circulation. The evidence therefore is that the mAb is inducing tolerance by an effect on antigen processing.

10 **12. Human desensitization protocol**

12.1 mAb or CDR peptides alone or with alum

Desensitization will optimally be started before the beginning of the allergy season. This will vary depending on the location, climate and housing facilities. Thus, for example, in locations in which symptoms began in May and October, desensitization would begin in the month prior to these two seasons. Subjects would be those people who clinically suffer from allergy to DMA as demonstrated by symptoms, and positive skin test. They will be immunized with mAb/CDR peptides, administered singly as soluble protein, or as protein admixed with aluminum hydroxide gel (such as "ALHYDROGEL 85", provided by SuperPhos Biosector a/s Vedbaek Denmark). The protein content of each injection will range between 1 μ g to 10 mg of protein, depending on the use of alum, the affinity of the antibodies/CDRs, and the number of components of the cocktails. Injections will be repeated weekly to monthly for up to six months, given subcutaneously (if mixed with alum), or either intradermally or subcutaneously (if no alum is used). Other adjuvants besides alum may also be used.

Subjects demonstrate significant reduction of symptoms as compared to controls. These symptoms depend on the type of allergy being treated. Patients with atopic dermatitis demonstrate decrease in area of skin involved, and/or amount of medication used. Those with

asthma showed decrease in cough and episodes of
breathlessness, decrease in daily medication, and
improvement in pulmonary function tests. Those with
allergic rhinitis showed decrease in number of months/
5 year requiring medication, and reduction in medication
required. In some cases the amount of allergen specific
IgE is decreased as measured in vitro, as is the
proliferation in vitro of T cells in response to antigen.
In vivo, the amount of allergen necessary to provoke a
10 positive skin test is increased, and the area of the
wheal and flare reaction at fixed dose of antigen is
decreased.

12.2 Antigen-antibody complexes

15 In another procedure, the mAb/CDR peptides may be
mixed with antigen, at allergen:mAb ratios from 1:2 to
1:10, injected into allergic patients weekly to monthly
for up to six months, given intradermally or
subcutaneously, at doses of mAb ranging from 1-20
20 μ g/dose. Reduction of symptoms are measured as described
above.

It will be apparent to those skilled in the art that
the present invention adds to the state of the art
antibodies, antigen-antibody complexes, and adjuvant
25 combinations effective in the treatment of human
allergies.

13. Deposit Information

On September 16, 1993, the following hybridomas were
30 deposited with the European Collection of Animal Cell
Cultures (ECACC):

Vaccine Research and Production Laboratory
Public Health Laboratory Service
Centre for Applied Microbiology and Research
Porton Down, Salisbury, Wiltshire
35 SP4 0JG United Kingdom

	<u>Hybridoma</u>	<u>ECACC</u> <u>No.</u>
1.	Anti-Der p I 1122/2C7/B3/A1G (mAb 2C7)	93091612
2.	Anti-Der p I 1102/H11/A10/E7 (mAb H11)	93091613
5 3.	Anti-Der p I 1148/1B12/D6/2A12 (mAb 1B12)	93091615
4.	Anti-Der p II 1154/2H5/1A10/1E7 (mAb 2H5)	93091614

10 All specific examples and procedures described herein are provided to exemplify the invention, and not for limitation.

All publications and patent applications mentioned herein are hereby incorporated by reference to the same extent as if each were specifically incorporated by reference individually.

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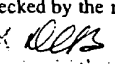
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International Application No: PCT/ /

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page __, lines __ of the description *	
A. IDENTIFICATION OF DEPOSIT *	
Further deposits are identified on an additional sheet *	
Name of depositary institution *	
European Collection of Animal Cell Cultures	
Address of depositary institution (including postal code and country) *	
Vaccine Research and Production Laboratory Public Health Laboratory Service Centre for Applied Microbiology and Research Porton Down, Salisbury, Wiltshire SP4 0JG United Kingdom	
Date of deposit * <u>September 16, 1993</u> Accession Number * <u>93091612</u>	
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office) <div style="text-align: right; margin-right: 100px;">  _____ (Authorized Officer) </div> <input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau * <div style="display: flex; justify-content: space-between; margin-top: 20px;"> <div>was</div> <div>_____</div> </div> <div style="text-align: right; margin-right: 100px;"> _____ (Authorized Officer) </div>	

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

European Collection of Animal Cell Cultures

Vaccine Research and Production Laboratory
Public Health Laboratory Service
Centre for Applied Microbiology and Research
Porton Down, Salisbury, Wiltshire SP4 0JG
United Kingdom

Accession No.

93091613

93091614

93091615

Date of Deposit

September 16, 1993

September 16, 1993

September 16, 1993

WHAT IS CLAIMED IS:

1. A method for suppression of an allergic response to an exogenous allergen, comprising administering to an animal a noncellular immunosuppressive composition comprising an isolated and purified antibody-derived or TCR-derived molecule which is capable of binding selectively *in vitro* to an allergenic epitope of said allergen, said composition being substantially free of said allergen, thereby selectively suppressing the allergic response of said animal to said allergen.
2. The method of Claim 1 wherein said allergic response comprises production of IgE antibodies against said allergen.
3. The method of Claim 2 wherein said allergic response comprises asthma.
4. The method of Claim 2 wherein said allergic response comprises allergic rhinitis.
5. The method of Claim 2 wherein said allergic response comprises conjunctival inflammation.
6. The method of Claim 2 wherein said allergic response comprises atopic dermatitis.
7. The method of Claim 1 wherein said allergenic epitope comprises an immunodominant epitope.
8. The method of Claim 1 wherein said animal is sensitized to said exogenous allergen by airborne exposure.

9. The method of Claim 1 wherein said exogenous allergen is a tree or broadleaf weed pollen, a mold spore, or a dust mite antigen.
- 5 10. The method of Claim 1 wherein said exogenous allergen is a food protein or a drug.
11. The method of Claim 1 wherein said animal is sensitized to said exogenous allergen prior to
10 administration of said immunosuppressive composition.
12. The method of Claim I wherein said immunosuppressive composition is substantially free of Ab2s to said
15 allergen.
13. The method of Claim 1 wherein said immunosuppressive composition is substantially free of antibodies other than Ab1s against said allergen.
- 20 14. The method of Claim 1 wherein said immunosuppressive composition comprises at least two Ab1s against different epitopes of said allergen.
- 25 15. A method for obtaining an immunosuppressive composition containing an antibody-derived molecule which binds selectively to an exogenous allergen, comprising the steps of:
- 30 a) selection of a monoclonal antibody which recognizes an immunodominant epitope on said allergen; and either
- b) demonstration that the selected monoclonal antibody stimulates an anti-idiotypic antibody which blocks binding of said monoclonal
35 antibody to said allergen, and demonstration that an anti-idiotypic antibody which blocks

- binding of said monoclonal antibody to said allergen is present in a patient receiving antigen immunotherapy with said allergen; or
- 5 c) demonstration that the selected antibody binds to an anti-idiotypic antibody induced in a patient receiving antigen immunotherapy with said allergen.
- 10 16. A noncellular composition for suppression of an allergic response to an exogenous allergen, comprising an isolated and purified antibody-derived or TCR-derived molecule which is capable of binding selectively *in vitro* to an allergenic epitope of said allergen, in a pharmaceutically acceptable
- 15 injectable solution said composition being substantially free of said allergen, said composition selectively suppressing the allergic response to said allergen when said composition is administered to said animal.
- 20 17. The composition of Claim 16 wherein said antibody-derived molecule is an antibody fragment.
- 25 18. The composition of Claim 16 wherein said antibody-derived molecule lacks an Fc domain.
- 30 19. The composition of Claim 16 wherein said exogenous allergen is a tree or broadleaf weed pollen, a mold spore, or a dust mite antigen.
- 35 20. The composition of Claim 16 wherein said exogenous allergen is a food protein or a drug.
21. The composition of Claim 16 wherein said composition is substantially free of antibodies other than Ab1s against said allergen.

22. The composition of Claim 16 wherein said antibody-derived molecule is a monoclonal antibody.

5 23. The composition of Claim 22 wherein said monoclonal antibody binds to *Der p I* at the binding site of antibody 2C7. and inhibits the binding of human serum IgE to *Der p I* by at least 40% in a majority of subjects allergic to *Der p I*.

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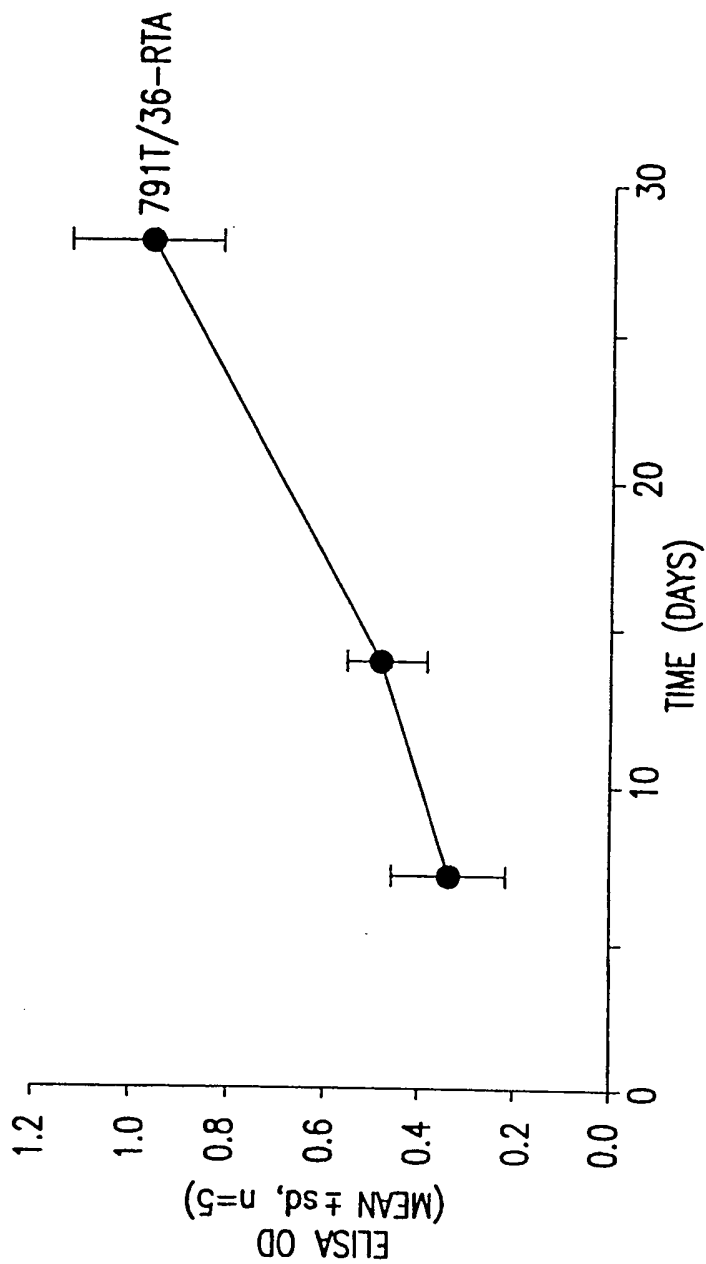
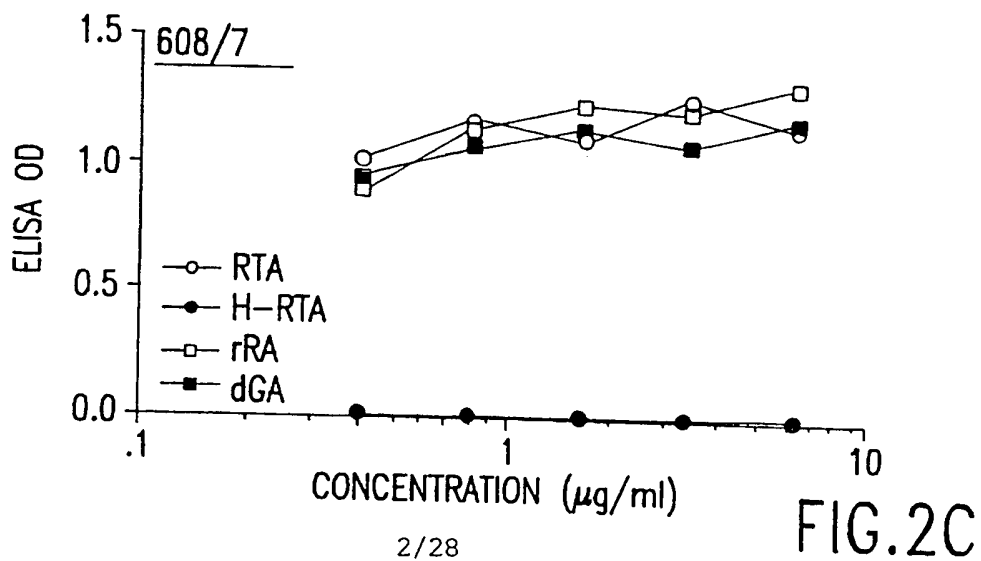
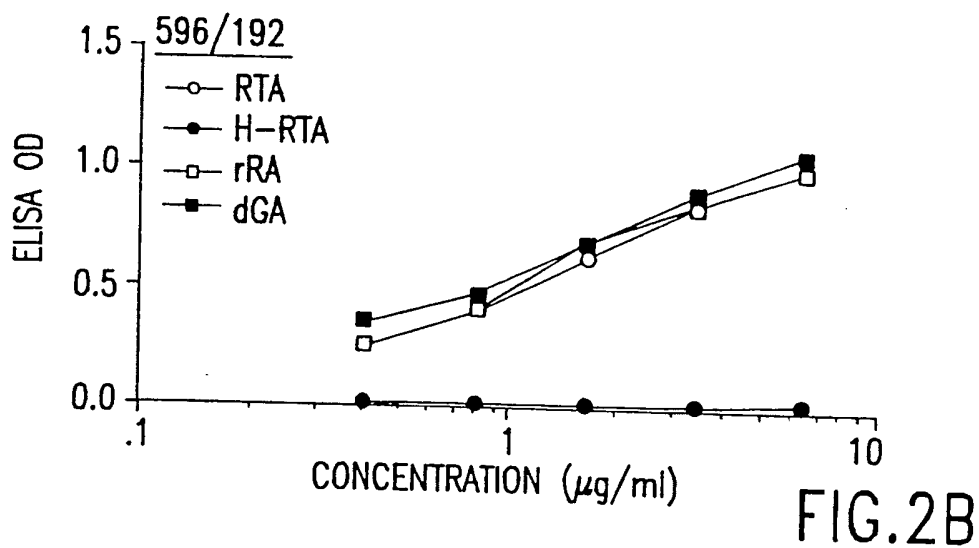
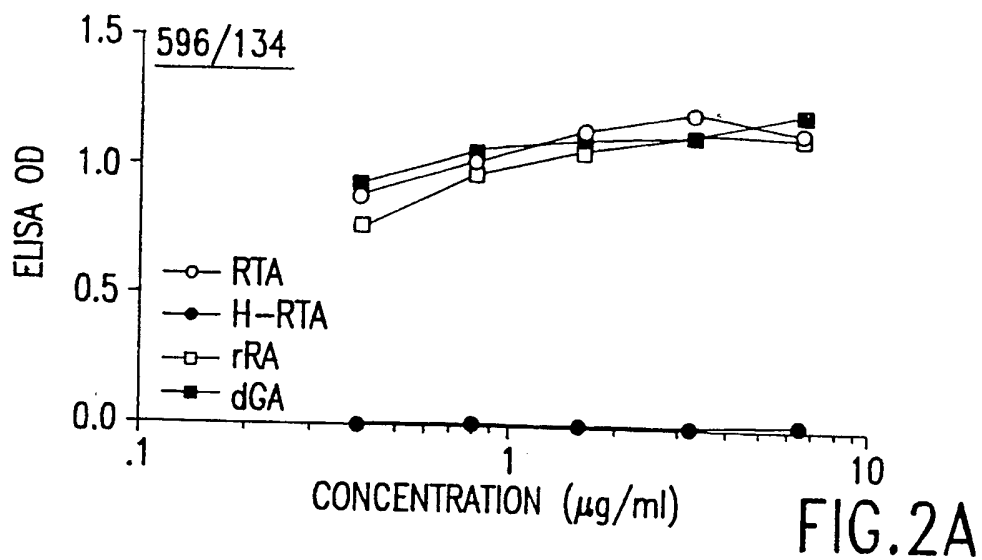
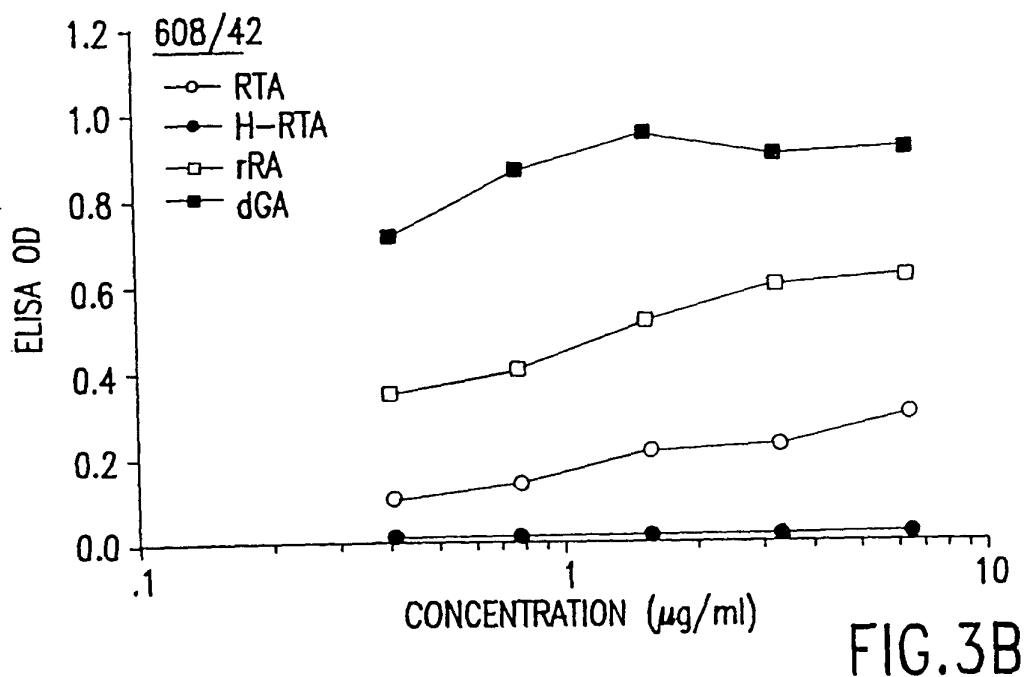
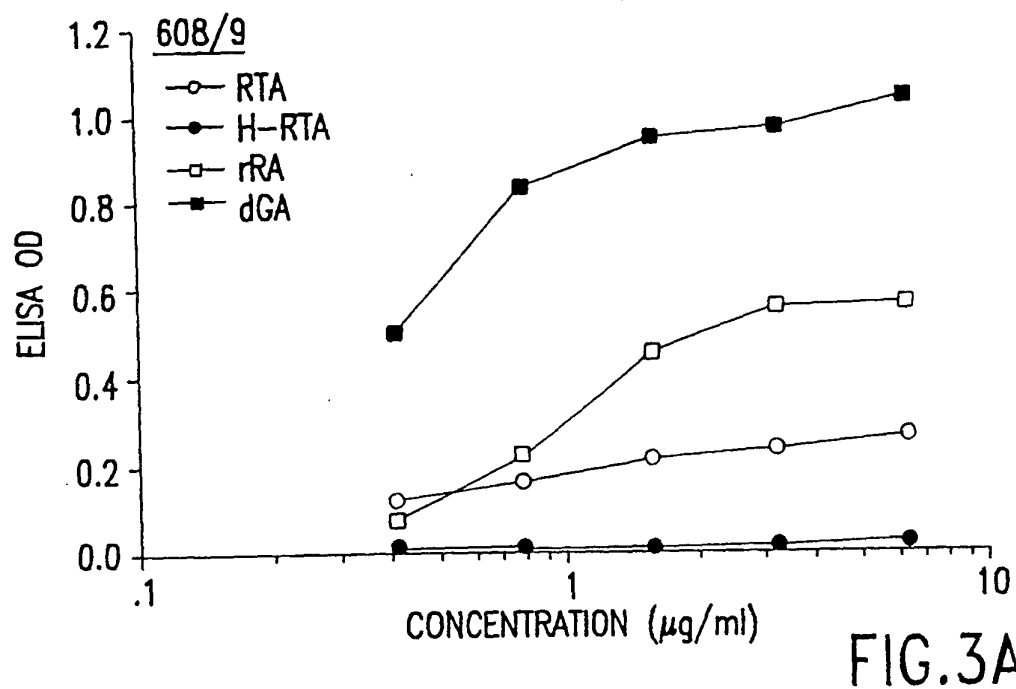


FIG.1





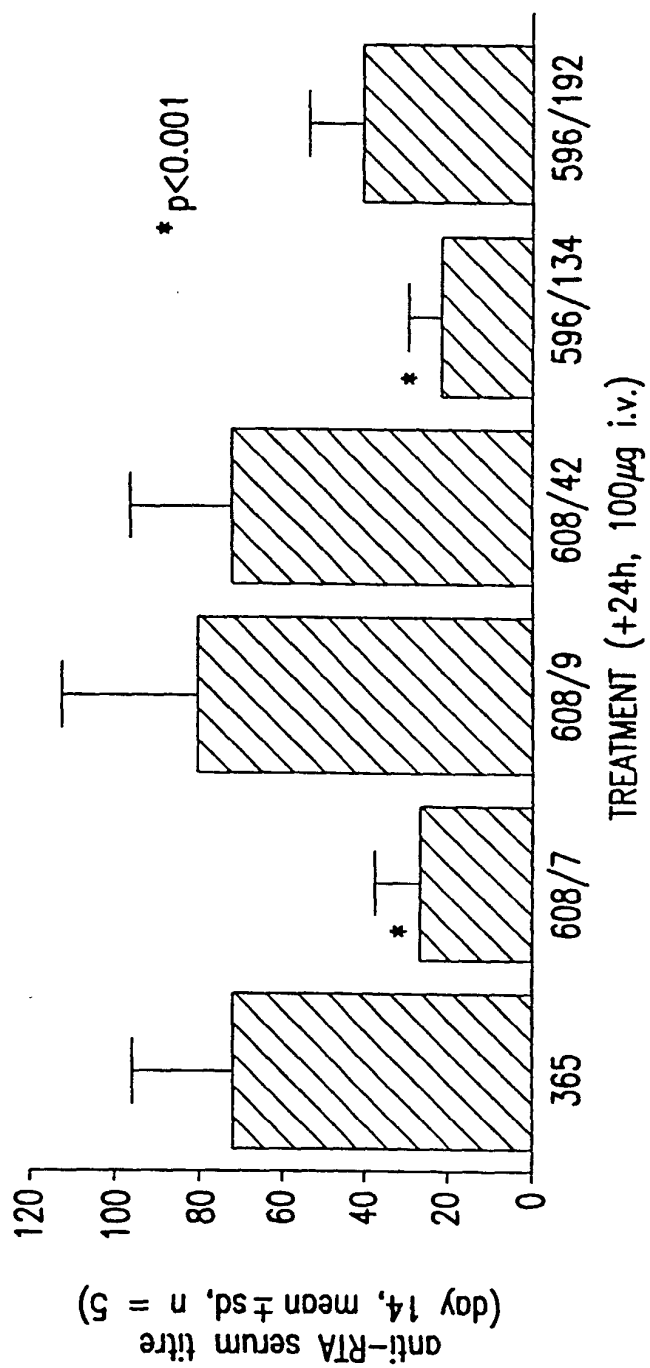


FIG. 4

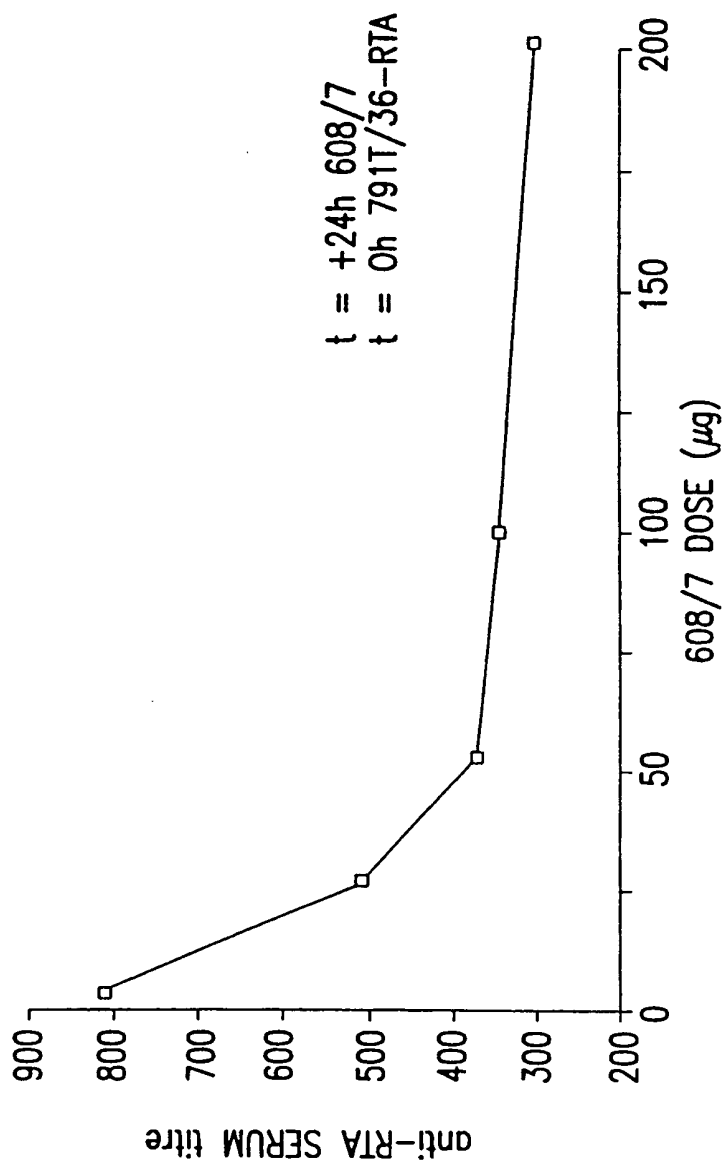


FIG.5

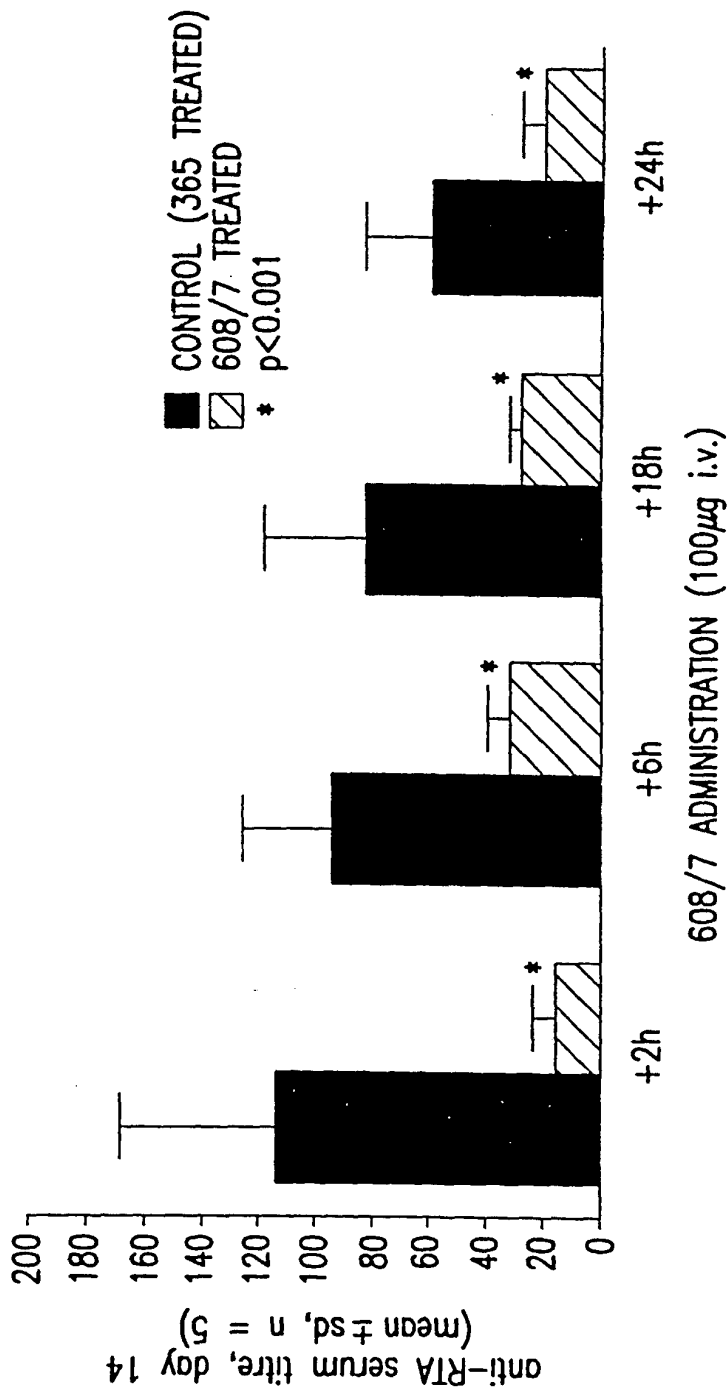


FIG. 6

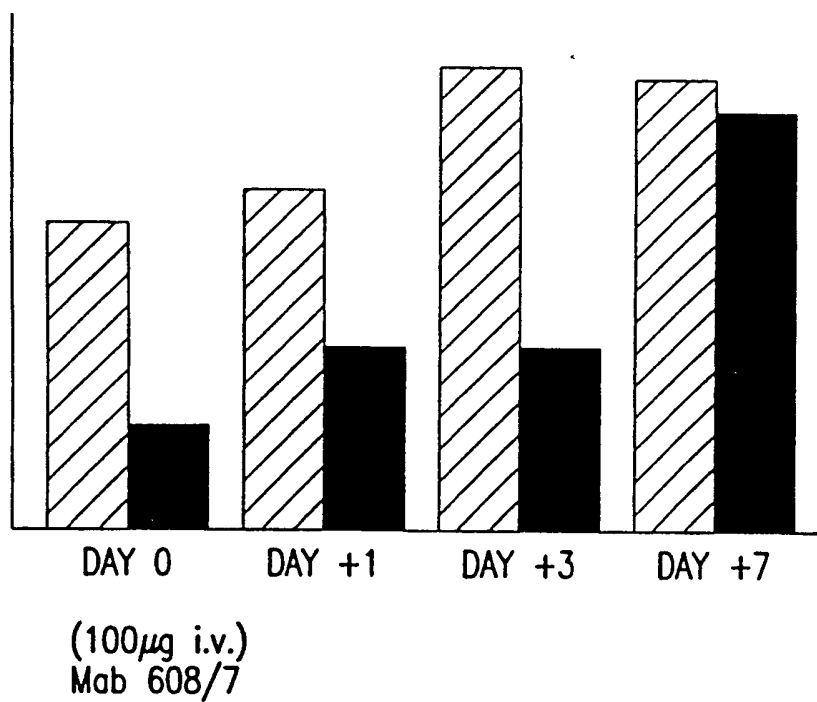


FIG.7

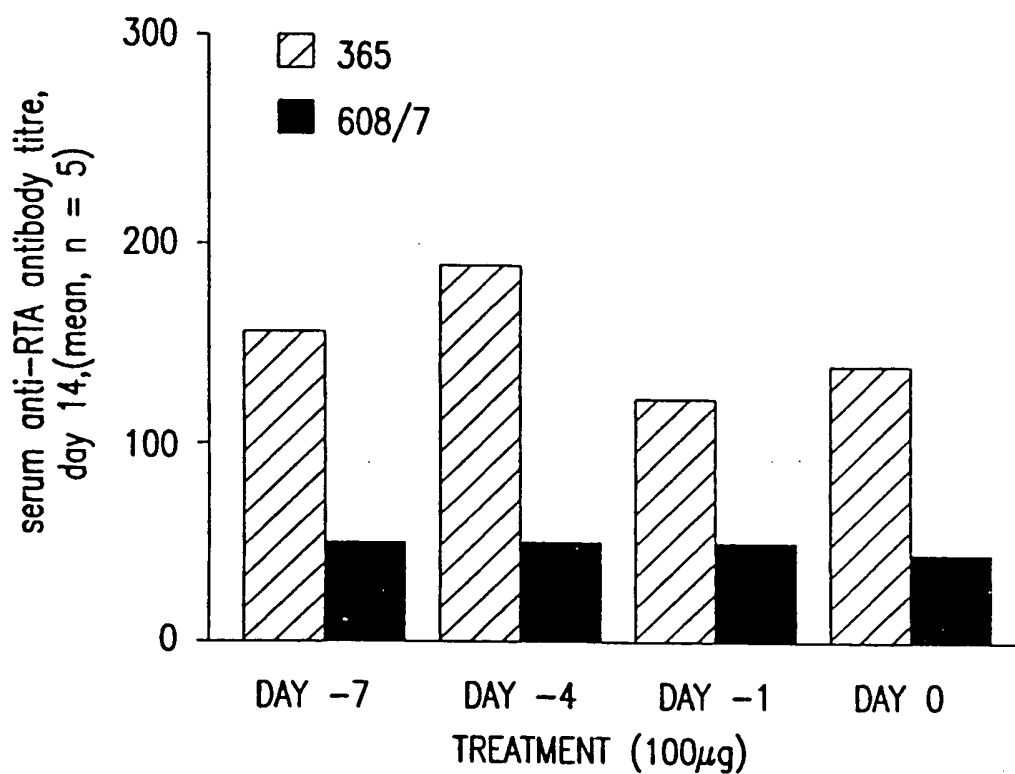


FIG.8

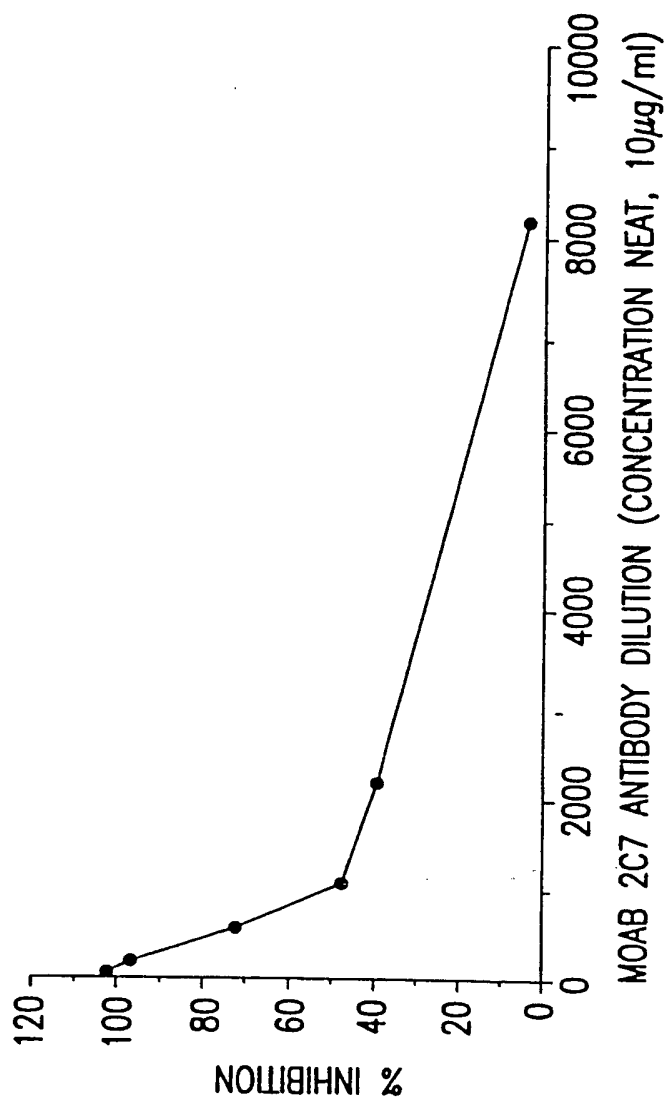
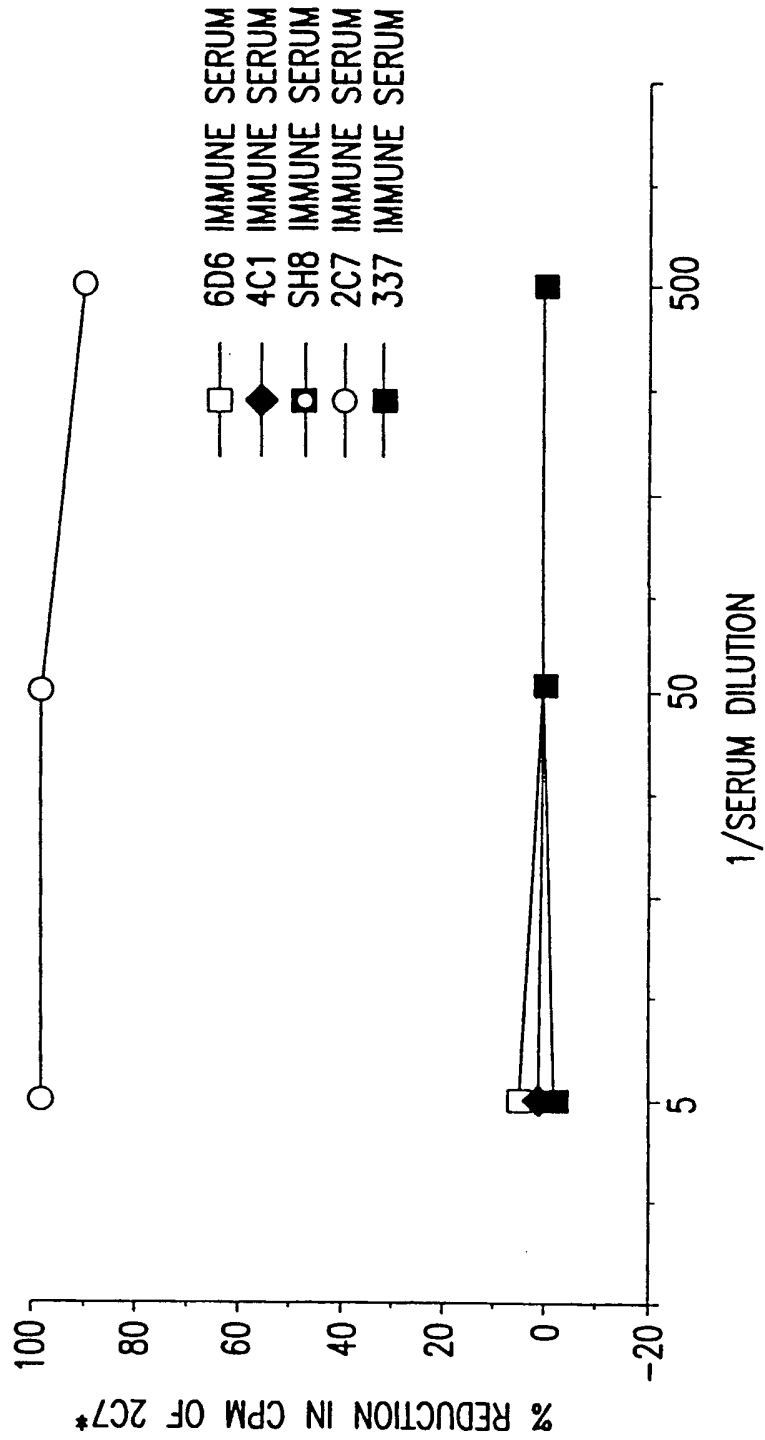


FIG.9



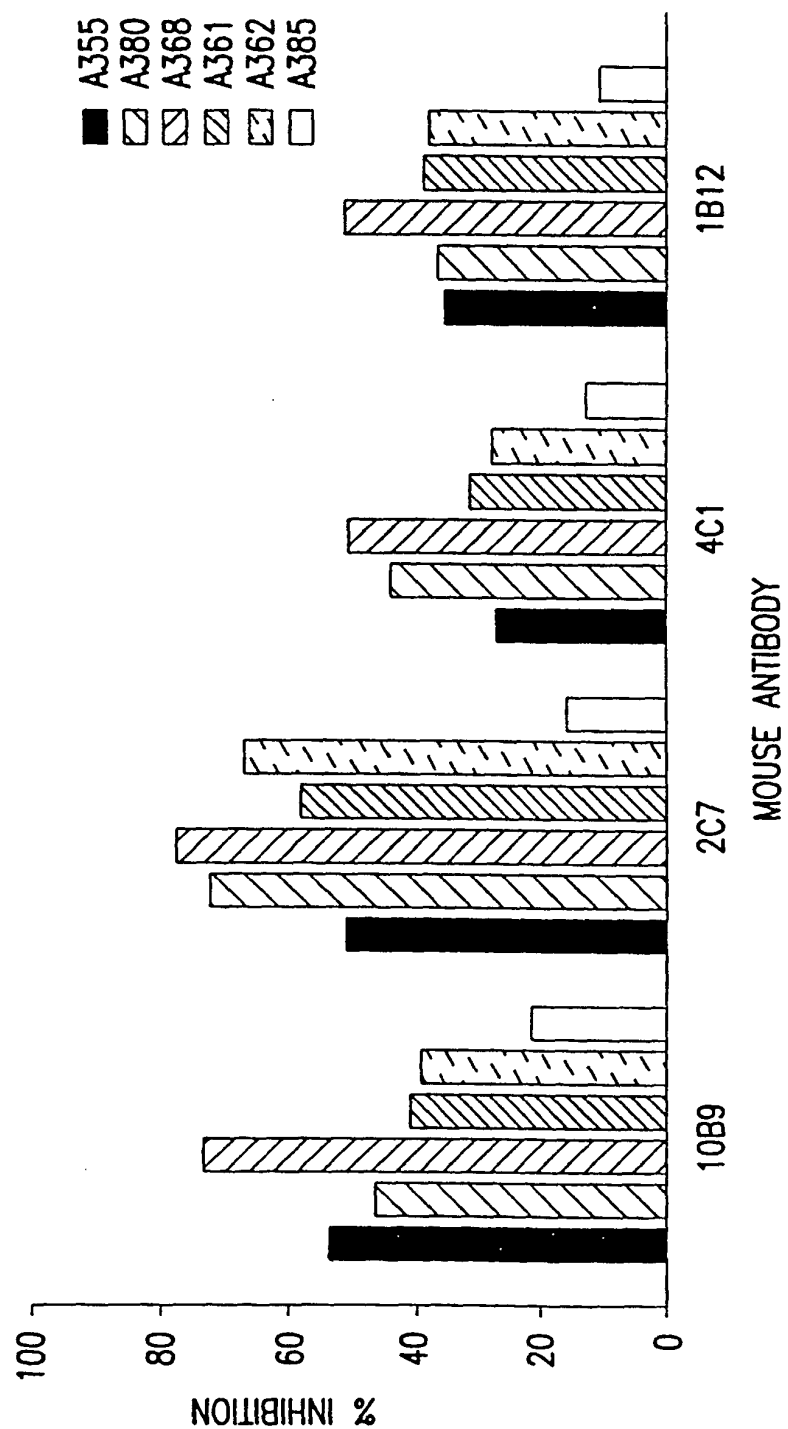


FIG.11

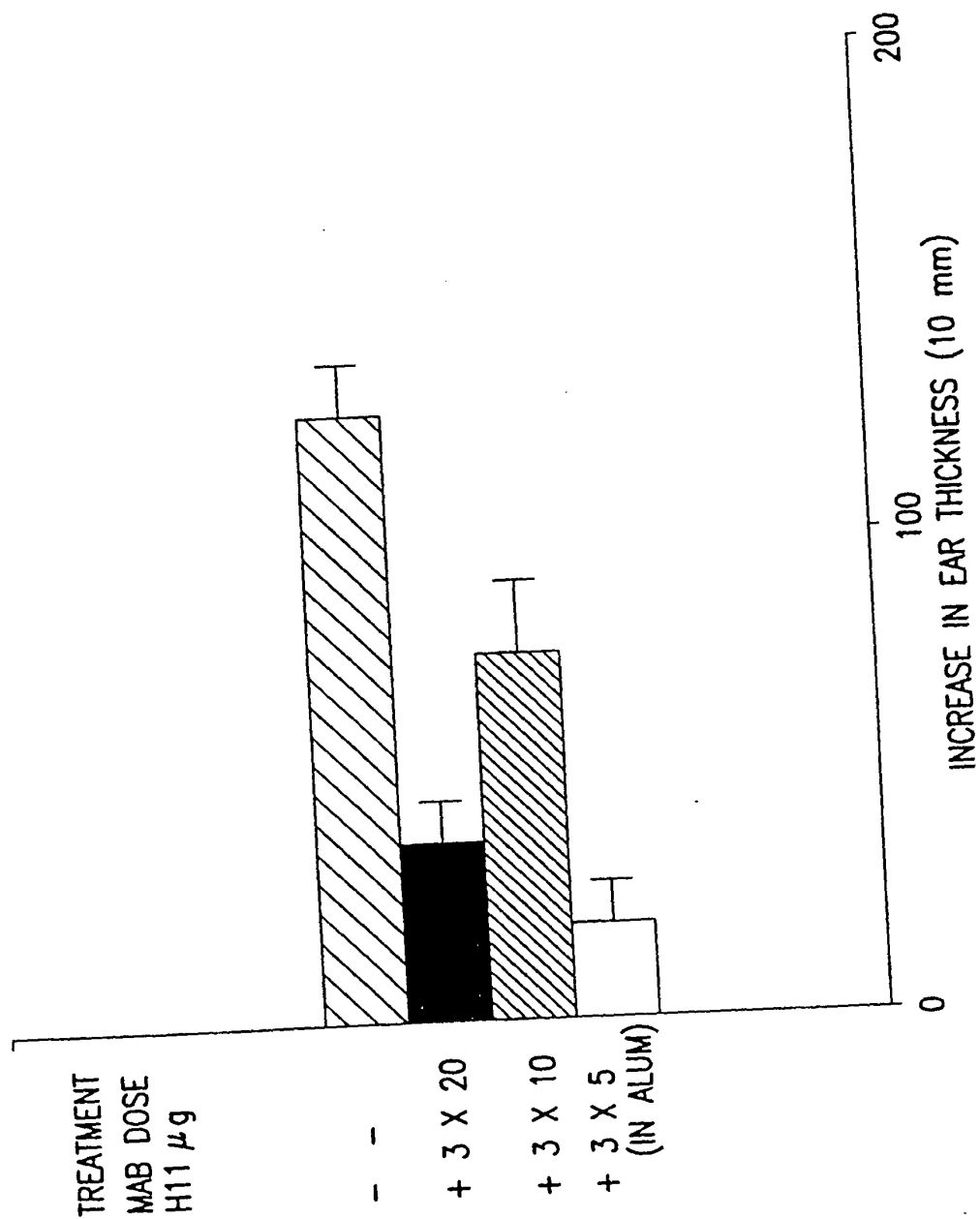


FIG.12

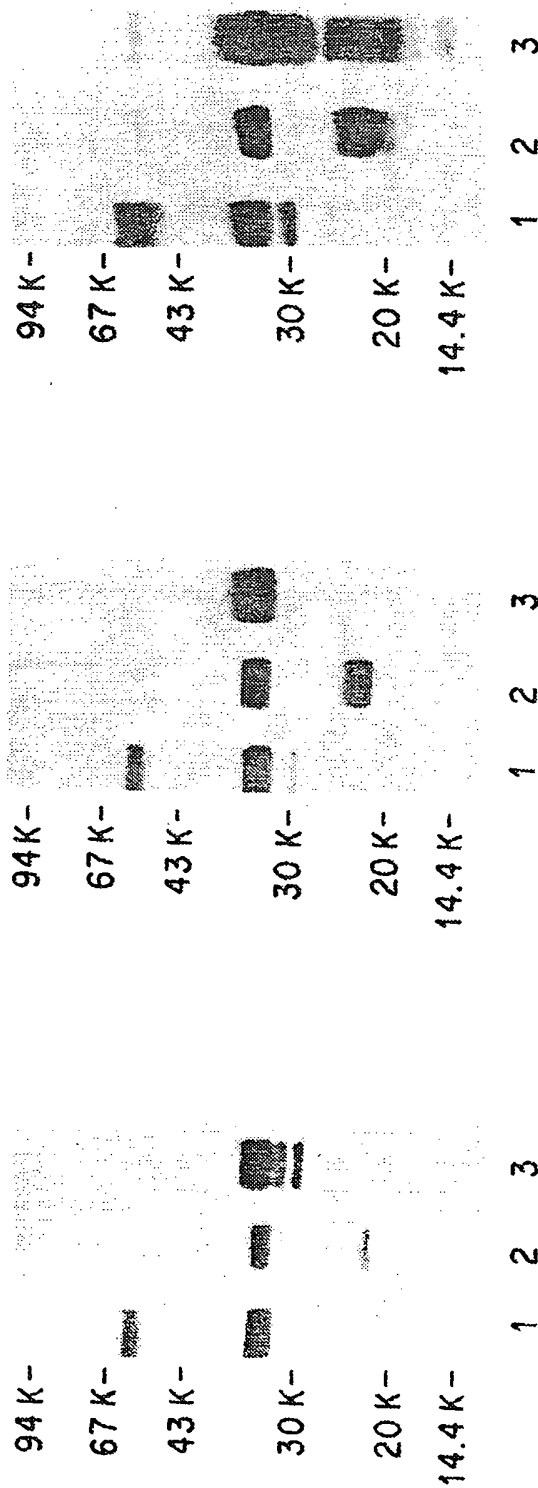


FIG. 13A

FIG. 13B

FIG. 13C

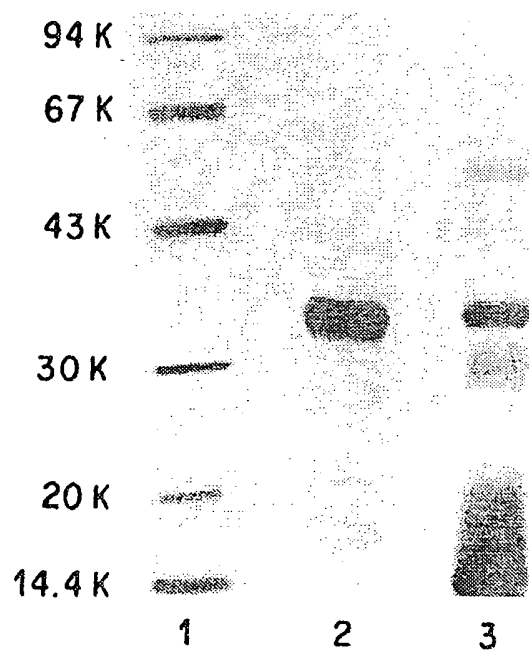


FIG. 14

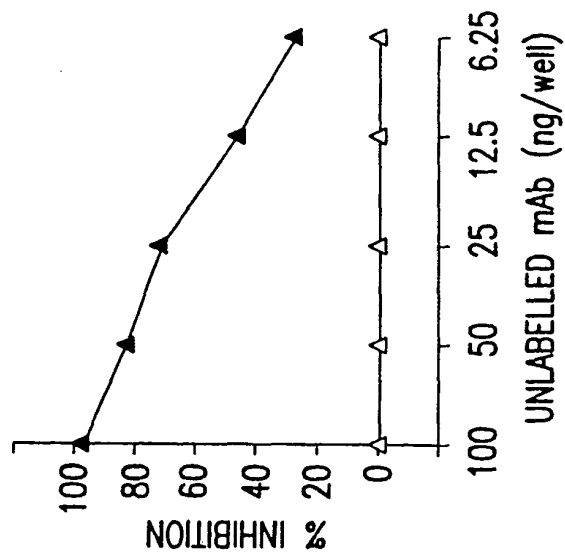


FIG. 15A

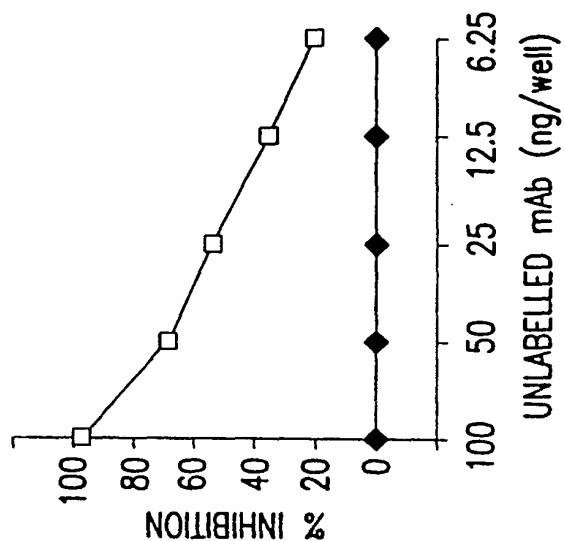


FIG. 15B

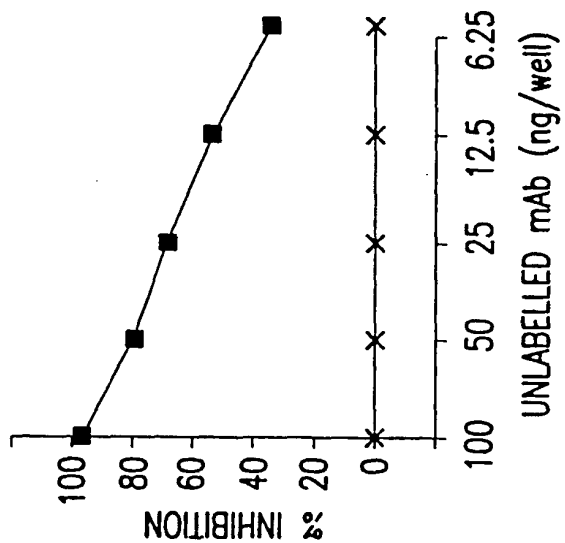


FIG. 15C

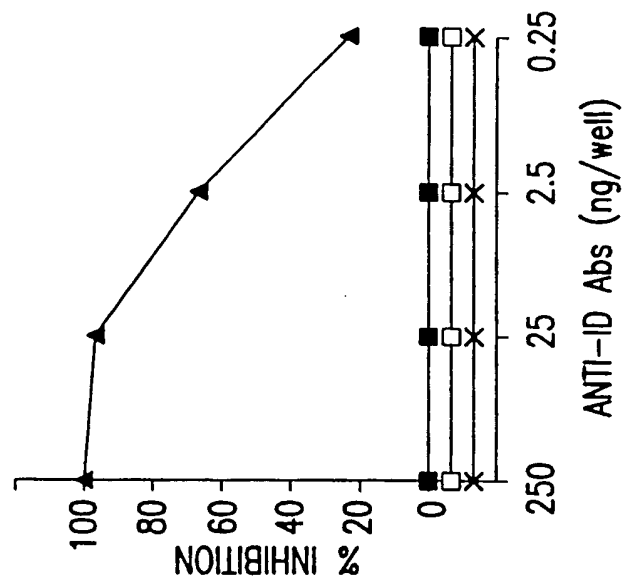


FIG. 16A

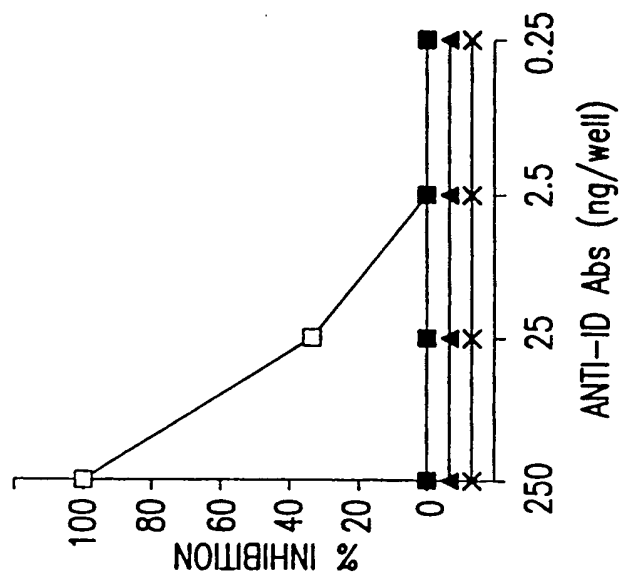


FIG. 16B

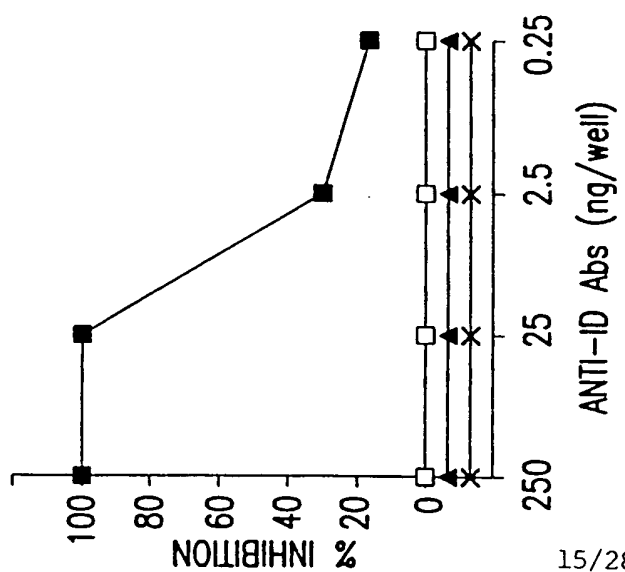


FIG. 16C

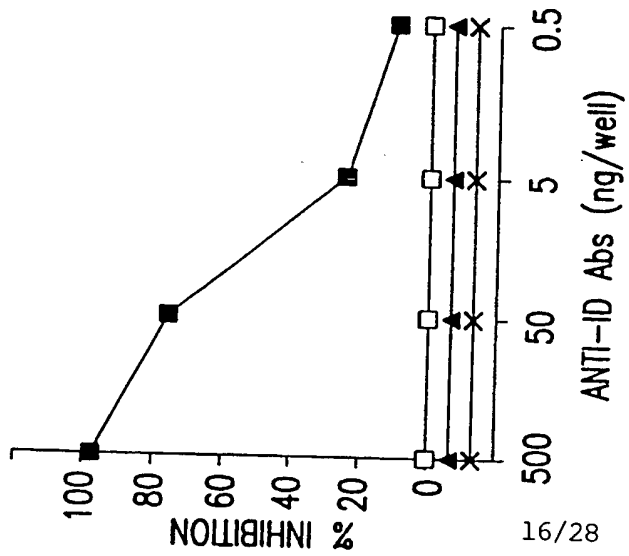


FIG. 17A

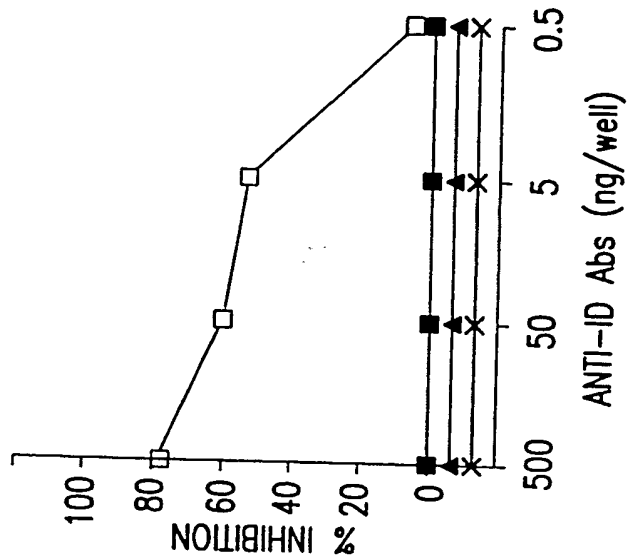


FIG. 17B

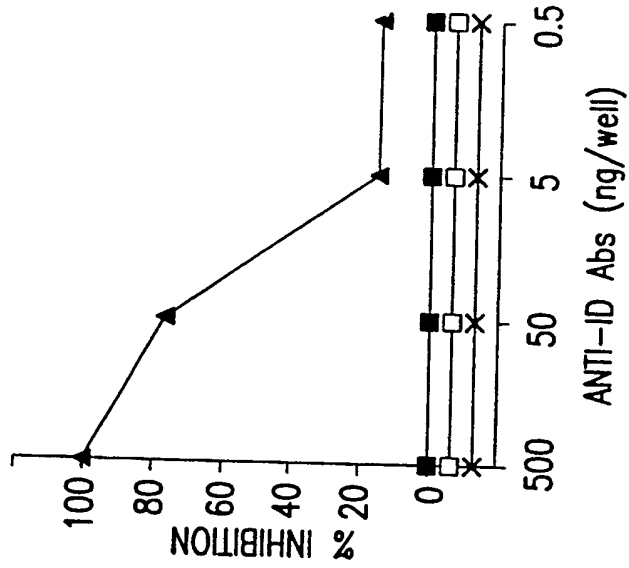


FIG. 17C

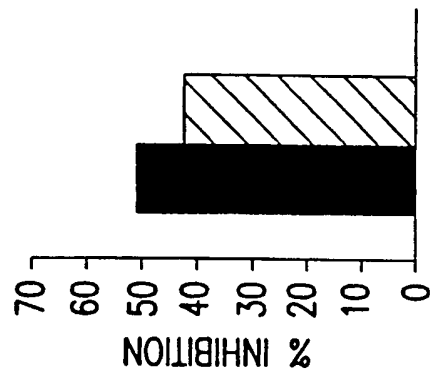


FIG. 18C

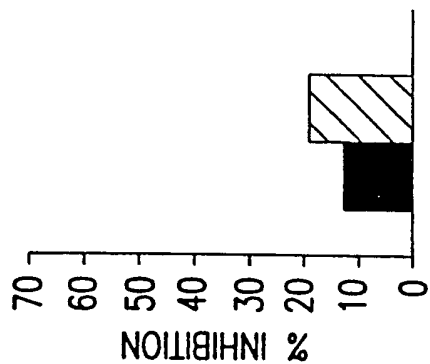


FIG. 18B

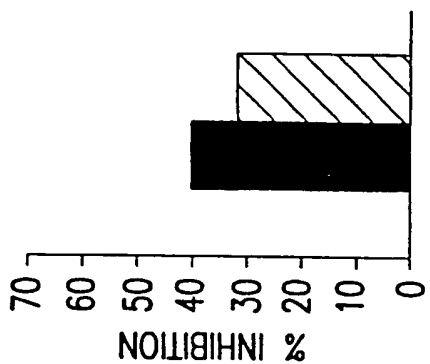


FIG. 18A

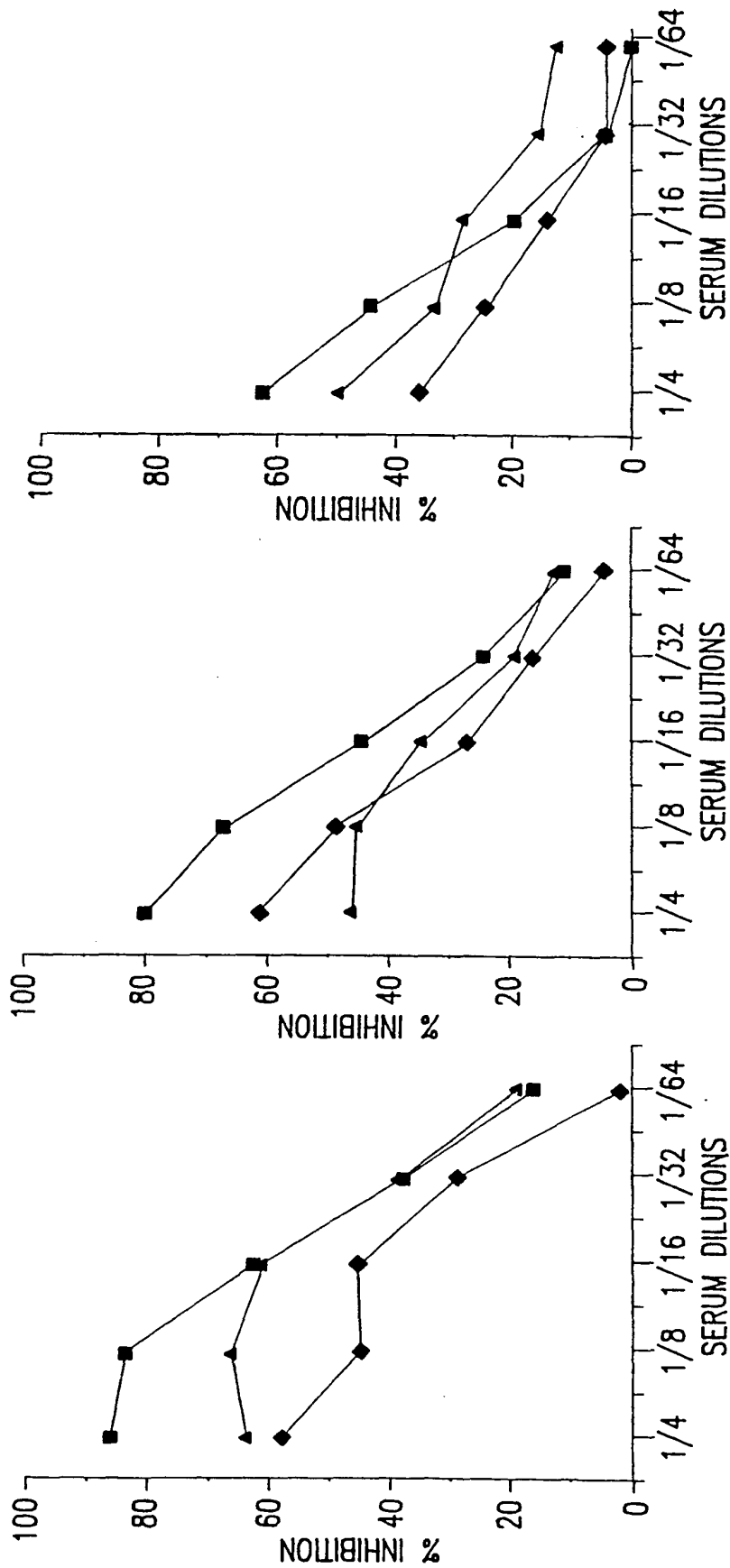


FIG.19C

FIG.19B

FIG.19A

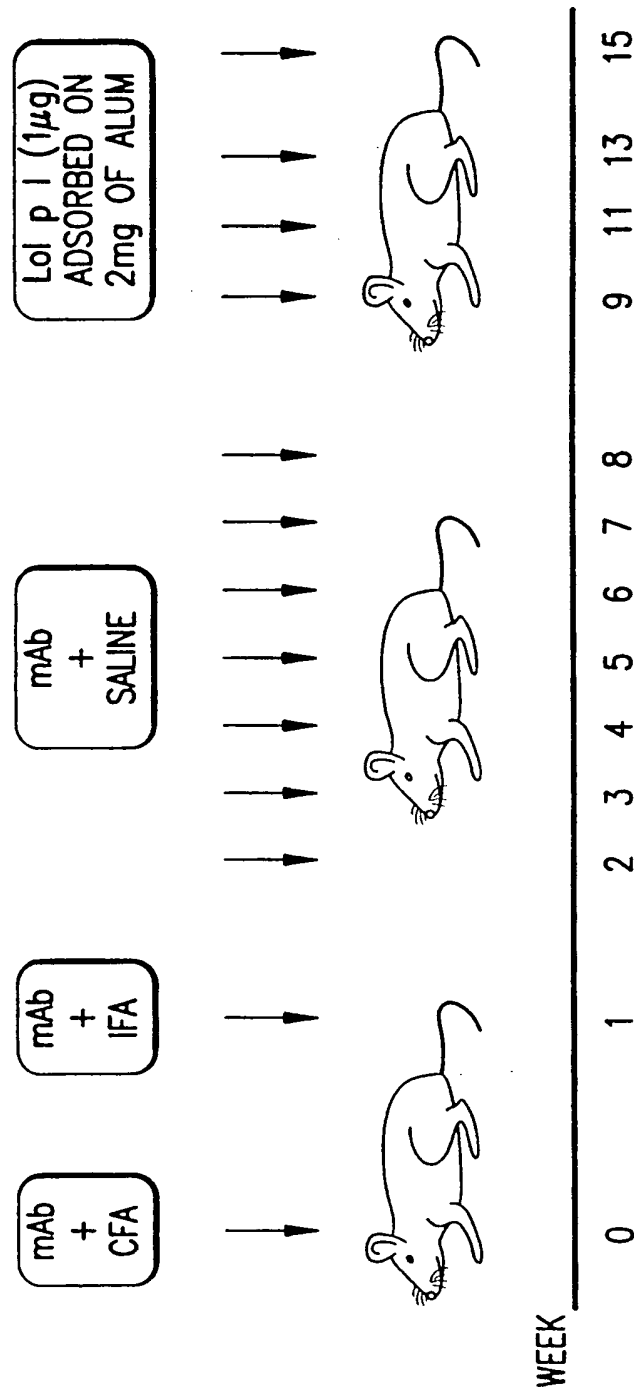


FIG.20

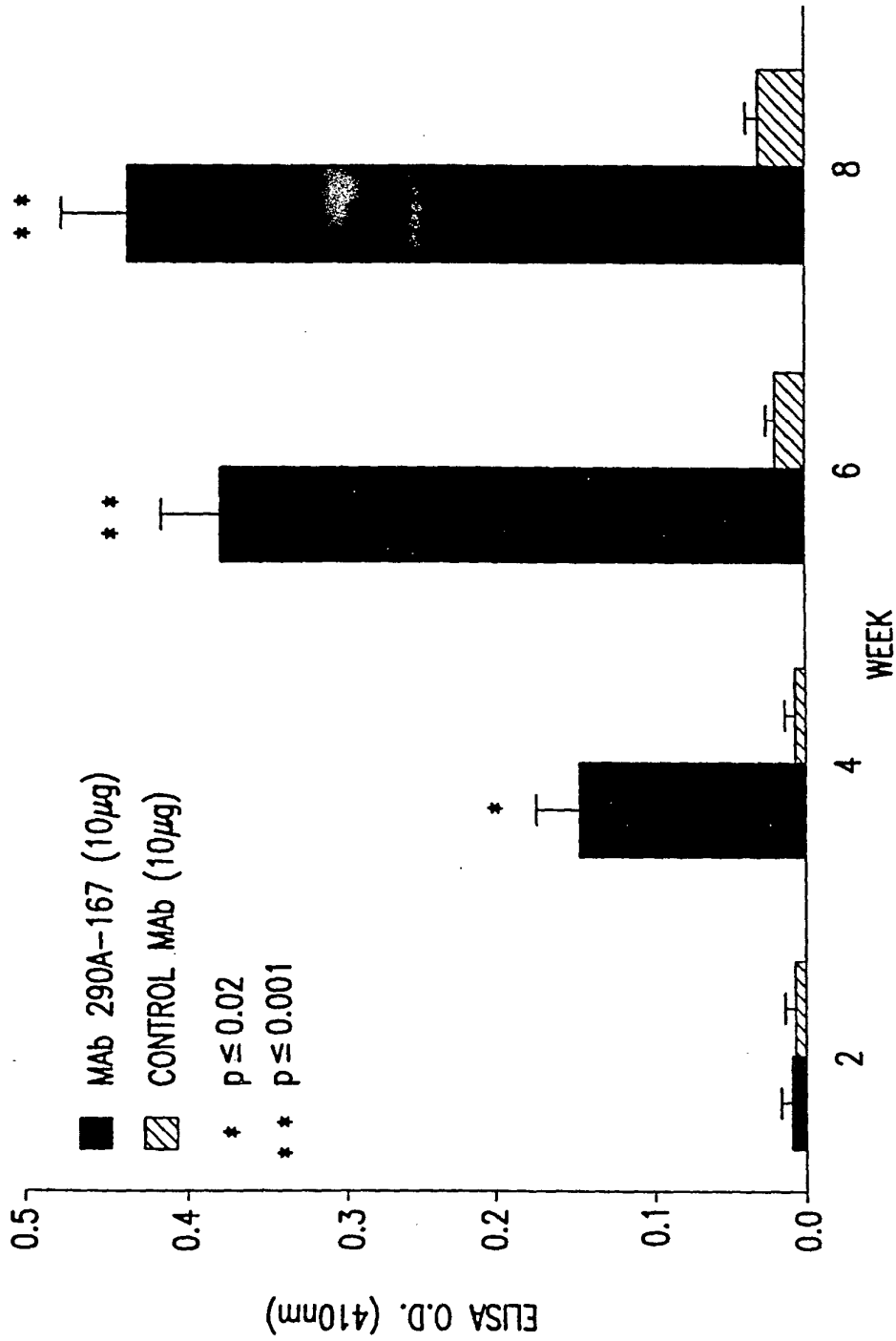


FIG.21

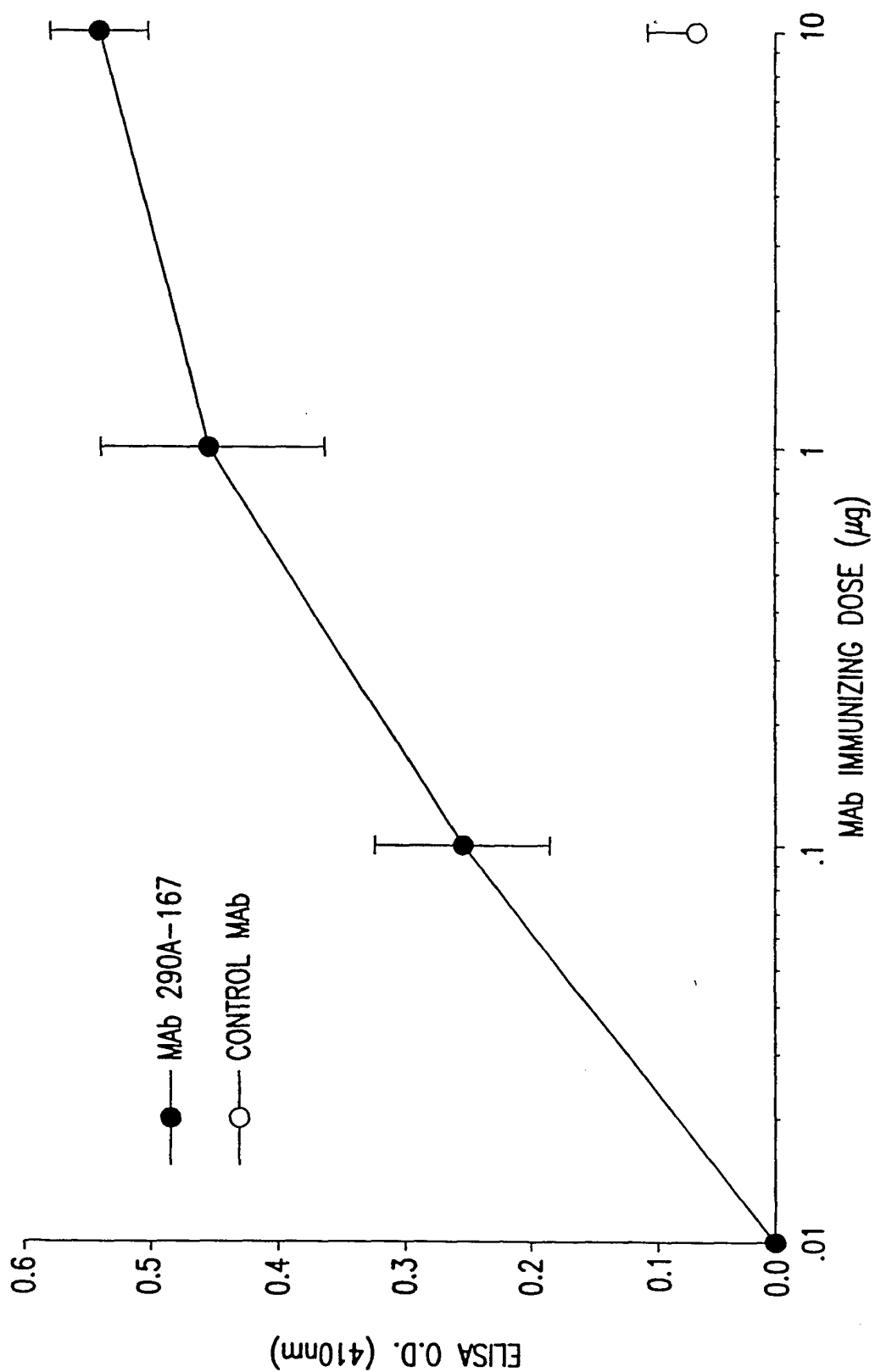


FIG.22

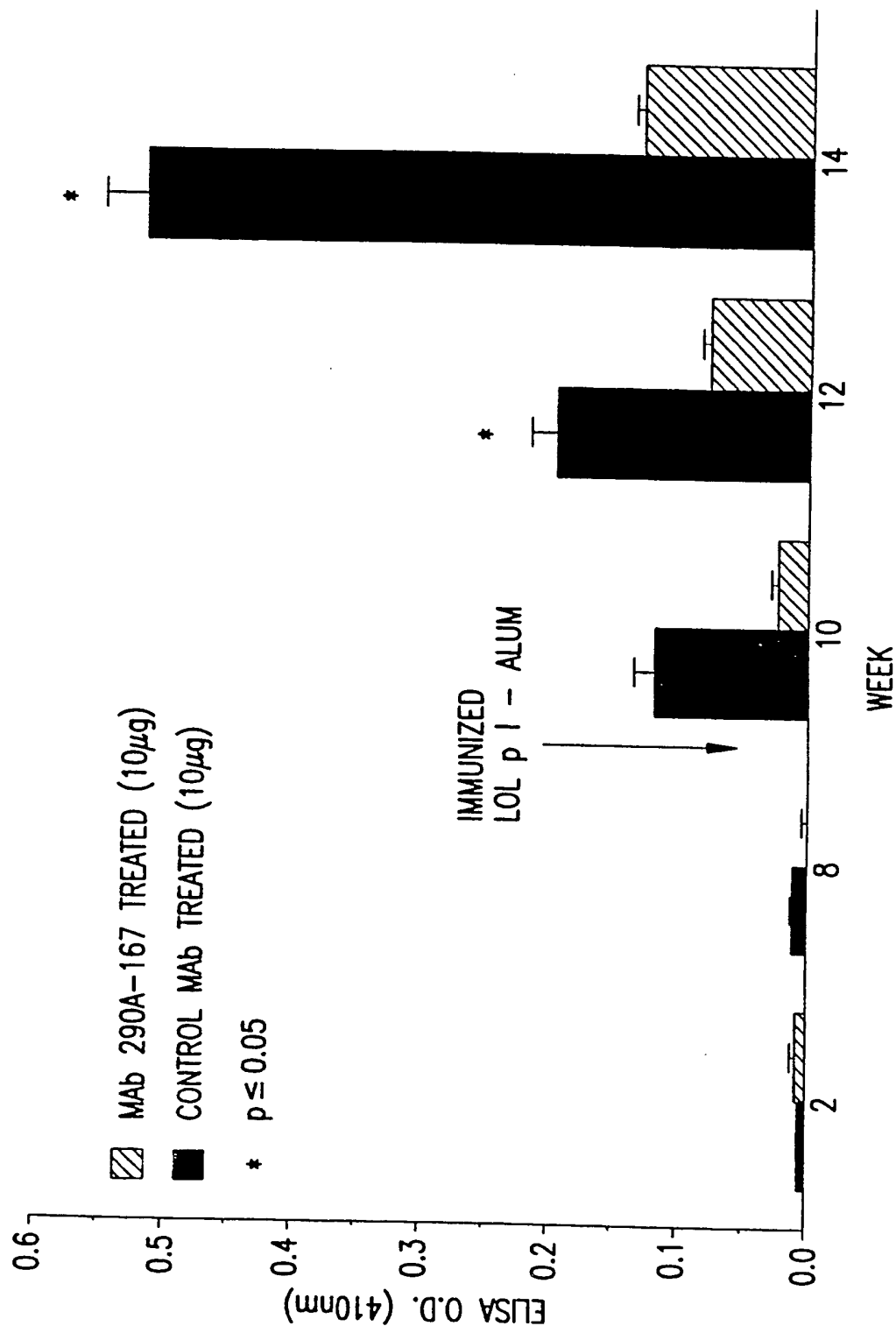


FIG.23

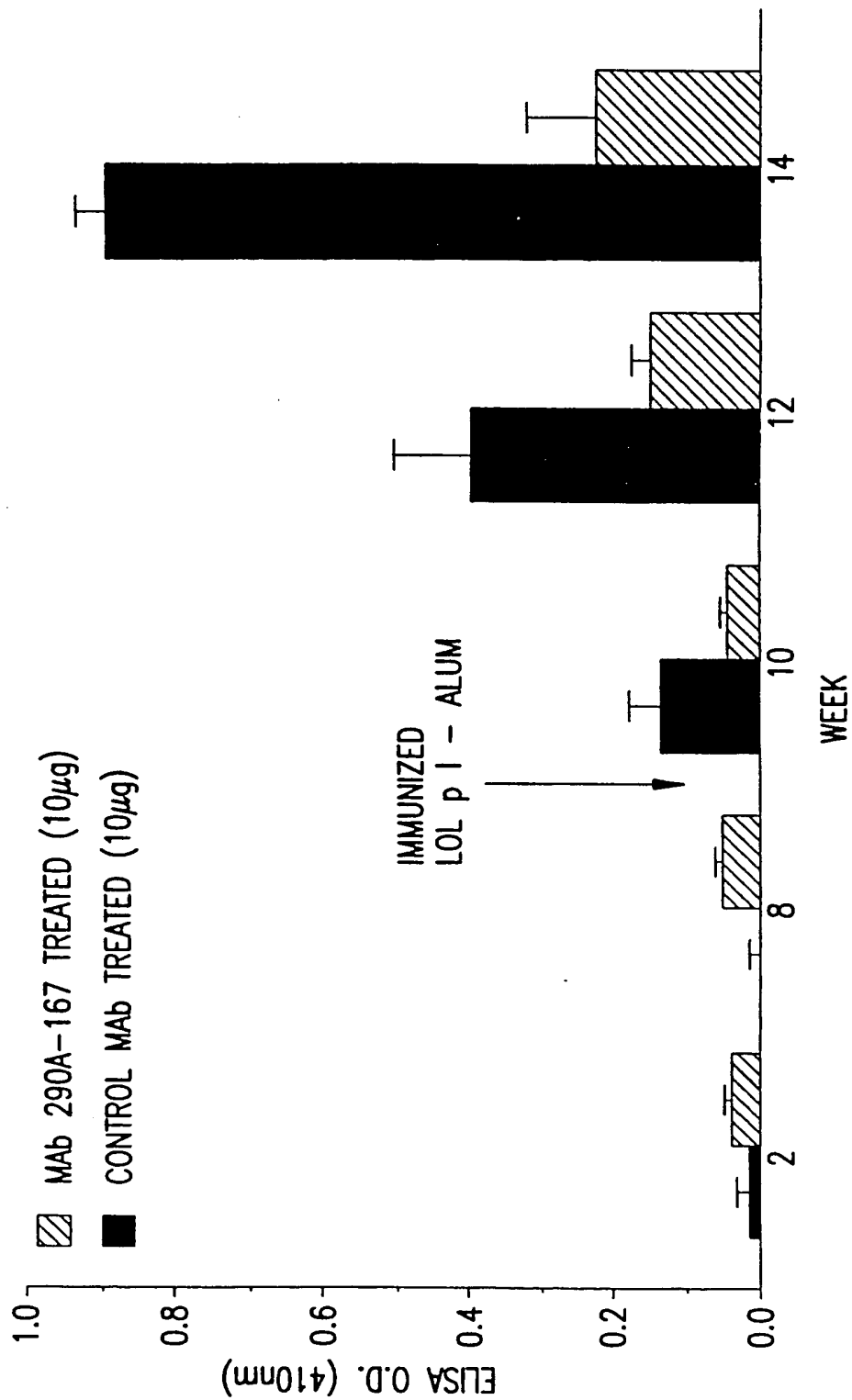


FIG.24

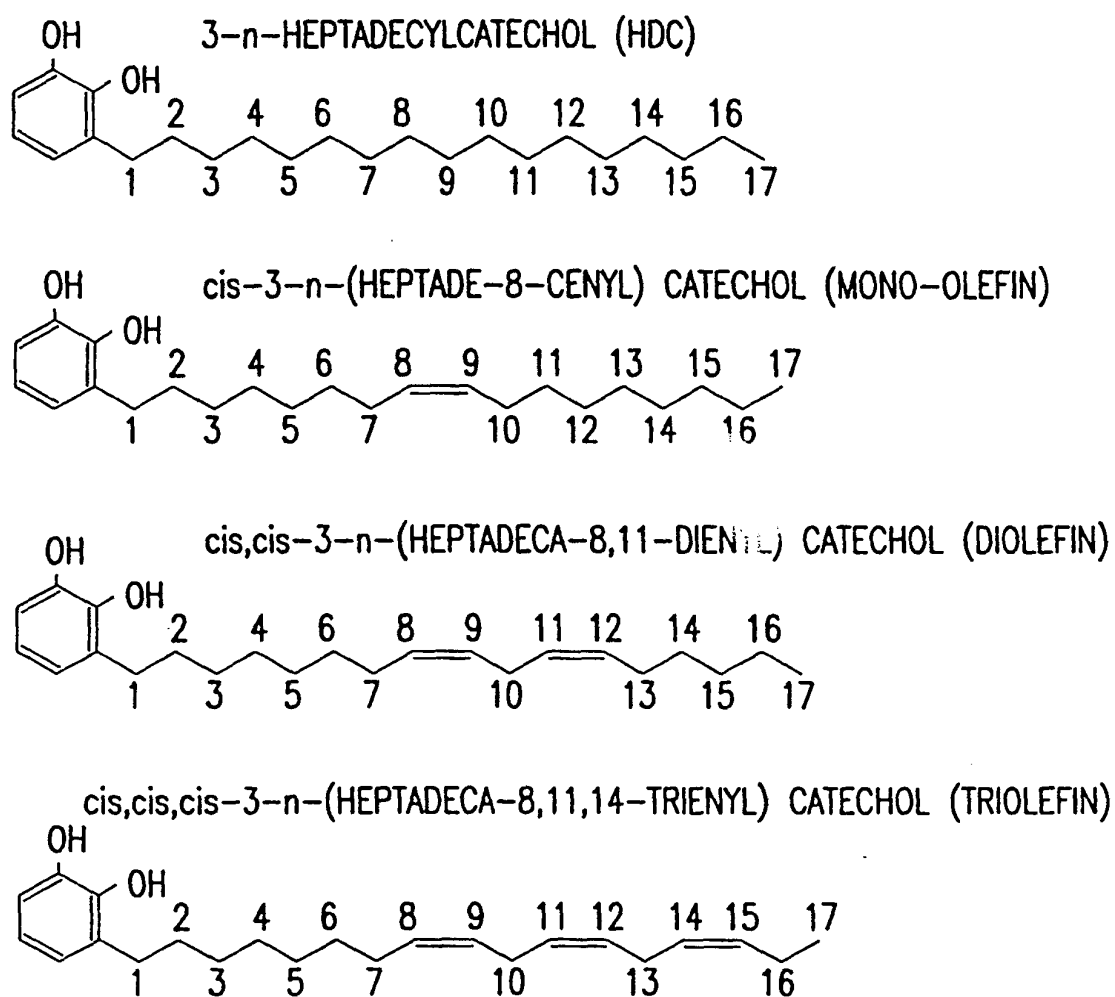


FIG.25

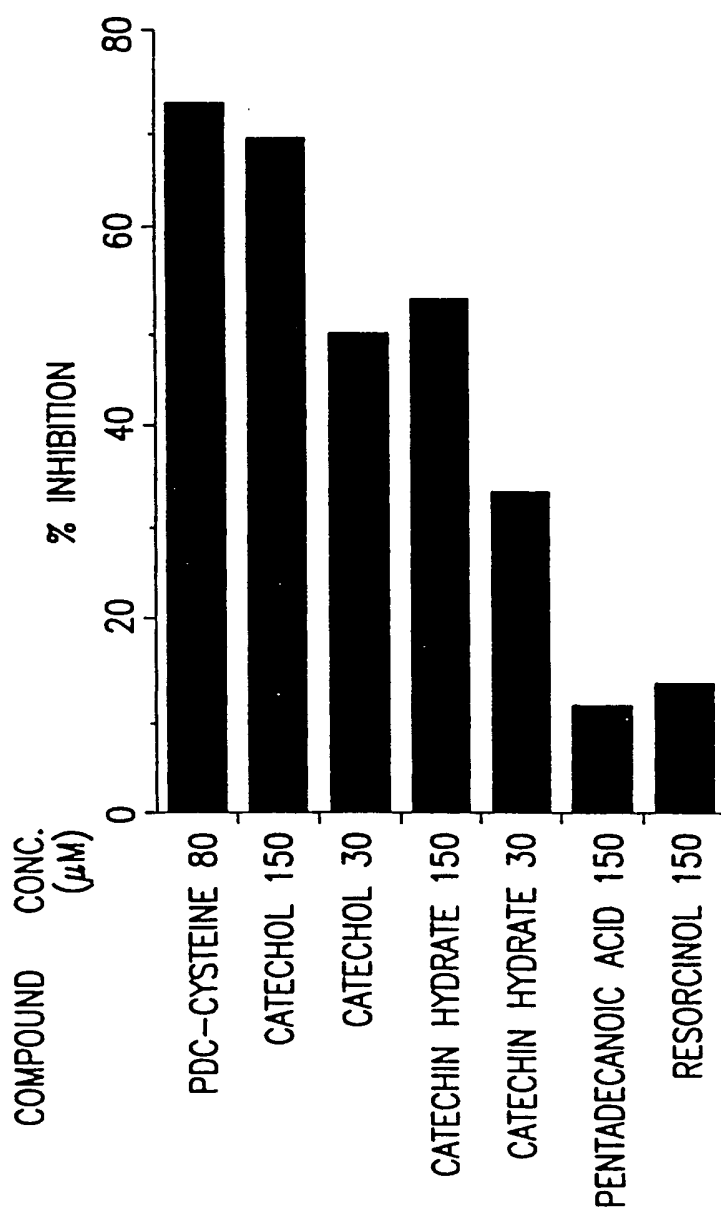


FIG. 26

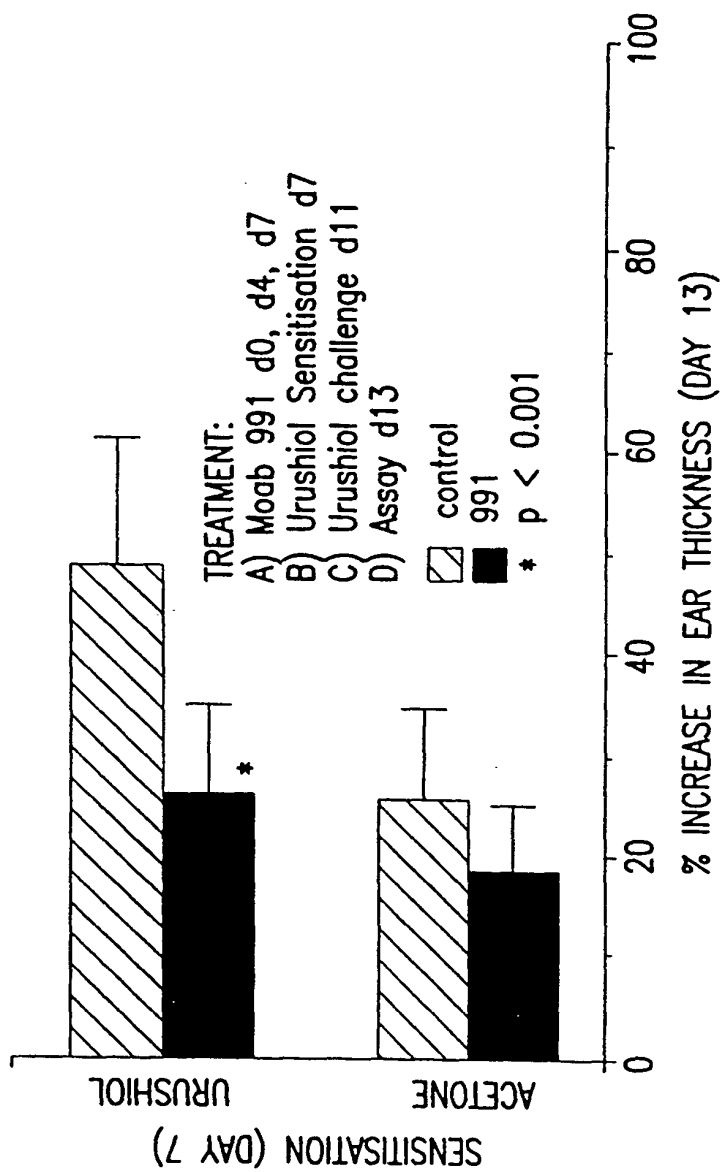


FIG.27

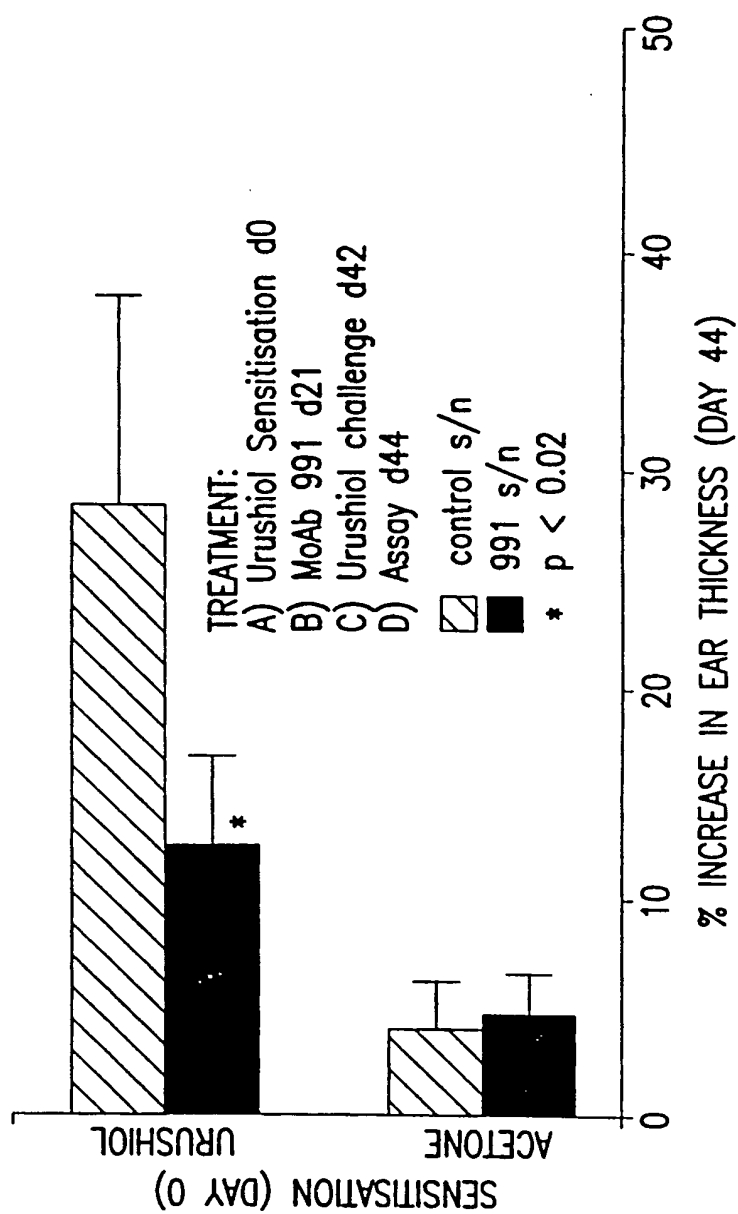


FIG.28

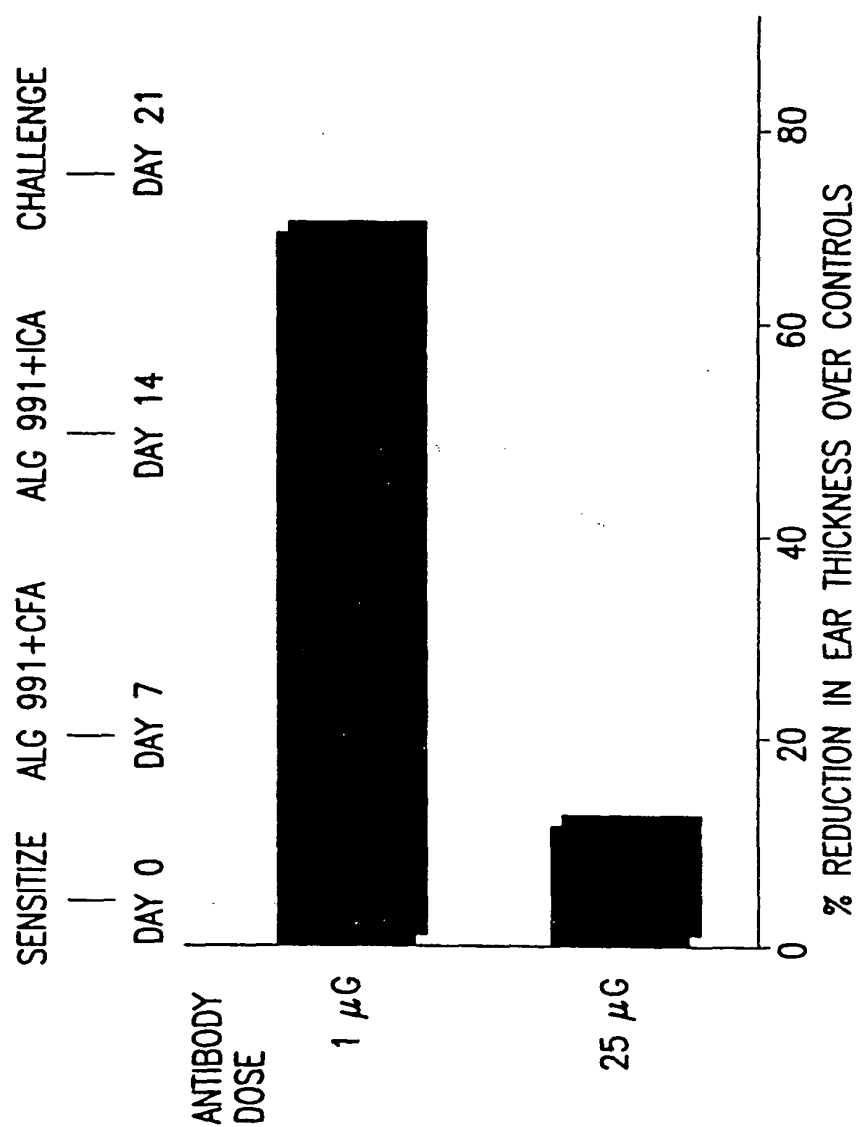


FIG. 29

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/10466

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 16/00; C12P 21/08; A61K 39/00

US CL : 530/388.22, 288.75, 389.7, 868; 424/130.1, 171.1, 184.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.22, 288.75, 389.7, 868; 424/130.1, 171.1, 184.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline

search terms: antibody, T-cell receptor, allergy, mold, pollen, food, drug, dust mite, Der p I

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Immunology, Volume 144, No. 4, issued 15 February 1990, Lombardero et al., "Conformational Stability of B cell Epitopes on Group I and Group II <i>Dermatophagoides</i> spp. Allergens", pages 1353-1360, see entire document.	1-23
Y	J. Investigative Dermatology, Volume 89, Number 3, issued September 1987, Dunn et al., "Regulation of Murine Contact Sensitivity to Urushiol Components by Serum Factors", pages 296-298, see entire document.	1-23



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	*G* document member of the same patent family

Date of the actual completion of the international search

01 DECEMBER 1994

Date of mailing of the international search report

22 DEC 1994

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/10466

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Allergy Clin. Immunol., Volume 77, Number 2, issued February 1986, Machtinger et al., "Cow's milk allergy in breast-fed infants: The role of allergen and maternal secretory IgA antibody", pages 341-347, see entire document.	1-23
Y	J. Immunology, Volume 139, Number 5, issued 01 September 1987, Chapman et al., "Epitope mapping of two major inhalant allergens, Der P I and Der F I, from mites of the genus dermatophagoides", pages 1479-1484, see entire document.	1-23
Y	Ann. Immunol. (Inst. Pasteur), Volume 125 C, issued 1974, Jerne "Towards a network theory of the immune system", pages 373-389, see entire document.	1-23
Y	Molecular Immunology, Volume 23, Number 12, issued 1986, Kahn et al., "Monoclonal antibodies to the major <i>lolium perenne</i> (rye grass) pollen allergen <i>Lol p I</i> (rye I)", pages 1281-1288, see entire document.	1-23
Y	J. Immunology, Volume 102, Number 1, issued 1969, Chang et al., "Studies on the mechanism of the suppression of active antibody synthesis by passively administered antibody", pages 37-41, see entire document.	1-23
Y	J. Immunology, Volume 138, Number 6, issued 15 March 1987, Eddy et al., "Idiotypic regulation of the anti-bovine serum albumin response I. Generation of an idiotype-specific Lyt-12 ⁺ suppressor T cell", pages 1693-1698, see entire document.	1-23
Y	J. Clin. Invest., Volume 82, issued September 1988, Kalish et al., "Urushiol (poison ivy)-triggered suppressor T cell clone generated from peripheral blood", pages 825-832, see entire document.	1-23
Y	TibTech, Volume 11, issued February 1993, Harris et al., "Therapeutic antibodies - the coming of age", pages 42-44, see entire document.	1-23